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Innate Modifiers of T Cell Behaviour During Inflammatory Disease

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Abstract

Neutrophils are the most abundant leukocyte in mammals and represent one of the first lines of defence against invading microorganisms. In recent years, it has become clear that neutrophils are not only responsible for the killing of pathogens, but that they also play an important role in shaping adaptive immune responses. The aim of this project was to determine the role of the neutrophil-derived host defence peptide, cathelicidin, in the generation of Th17 responses during inflammation. Following inoculation with heat-killed *Salmonella typhimurium*, cathelicidin knockout mice cannot produce IL-17 and show increased IFNγ, whereas other cytokines are produced normally. *Ex vivo*, I show that cathelicidin is a novel Th17/Tc17 differentiation enhancing factor, which acts directly on both $CD4^+$ and $CD8^+$ T cells to increase their activation status, protect them from death and concentration-dependently upregulate IL-17 production. Gene expression analysis revealed that cathelicidin downregulates the expression of several Th1-related genes and upregulates the aryl hydrocarbon receptor (AHR), a known regulator of Th17 differentiation. The addition of an AHR antagonist to our *in vitro* cultures abolishes the boost to IL-17 production normally induced by cathelicidin. I provide further evidence that suggests lymph node neutrophils are the cellular source of cathelicidin, which are responsible for amplifying type-17 responses during inflammation. These data contribute to our understanding of how lymph node neutrophils influence developing adaptive immune responses with sophistication and specificity.

Lay Summary

The immune system is typically divided into two categories: the innate and adaptive. The innate response consists of non-specific defence mechanisms that occur immediately after immune challenge, whereas the adaptive response occurs slightly later and displays great specificity. Cross talk between the two systems is critical for protecting the host against infection. The aim of this project was to determine how two different immune cells, neutrophils and T cells, interact and influence each other's behaviour. Neutrophils are the most abundant white blood cell type and constitute one of the first lines of defence as part of the innate immune response, whereas T cells are major players of adaptive immunity. More specifically, I sought to determine how a host defence peptide produced by neutrophils, called cathelicidin, is involved in driving the generation of a particularly damaging subset of T cells (Th17). Th17 cells are characterized by the production of IL-17, which has been shown to have many harmful effects, particularly during autoimmune disease. Mice that lack cathelicidin cannot produce IL-17 during inflammation. Here, I show that cathelicidin acts directly on the T cells, inducing several changes that lead to the development of Th17 features. Furthermore, I provide evidence that suggests neutrophil-derived cathelicidin acts on T cells and exerts its effects in a particular location: the lymph nodes. This data contributes to our understanding of how neutrophils influence the development of adaptive immune responses with sophistication and specificity.

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Declaration

I declare that this thesis is an original report of my research and has not been submitted for any other degree or professional qualification. The work contained herein is almost entirely my own, except where explicitly stated otherwise; collaborative contributions have been indicated clearly and acknowledged.

Name: Danielle Minns

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Abbreviations

3-MC: 3-methycholanthrene

AHR: aryl hydrocarbon receptor

AKT: protein kinase B

AMP: antimicrobial peptide

APC: antigen-presenting cell

APRIL: a proliferation-inducing ligand

AS: ankylosing spondylitis

Bac2A: bactenencin 2A

BAFF: B cell-activating factor

BAL: bronchoalveolar lavage

BATF: basic leucine zipper transcriptional factor ATF-like

BBB: blood brain barrier

BCG: Bacillus Calmette–Guérin

BCL: B cell lymphoma

CAMP: cathelicidin antimicrobial peptide

CCL: chemokine (C-C motif) ligand

CCR: chemokine (C-C motif) receptor

CD: Crohn's disease

CFA: complete Freund's adjuvant

CFSE: carboxyfluorescein succinimidyl ester

c-JNK: c-Jun N-terminal kinase

CLEC9A: C-type lectin domain family 9 member A

CNS: central nervous system

COPD: chronic obstructive pulmonary disease

CTLA4: cytotoxic T lymphocyte-associated protein 4

CXCL: chemokine (C-X-C motif) ligand

CXCR: chemokine (C-X-C motif) receptir

DAPI: 4',6-diamidino-2-phenylindole

DC: dendritic cell

DISC: death-inducing signalling complex

DLN: draining lymph node

DN: double negative

DNA: deoxyribonucleic acid

DP: double positive

DSS: dextran sodium sulphate

EAE: experimental autoimmune encephalomyelitis

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

Eomes: Eomesodermin

ER: endoplasmic reticulum

ERK: extracellular signal-related kinase

FADD: Fas-assocated death domain

FC: fold change

FCS: foetal calf serum

FICZ: 6-formylindolo[3,2-b]carbazole

fMLF: N-formylmethionyl-leucyl-phenylalanine

FMO: fluorescence minus one

FOXP3: forkhead box P3

FPR2: formyl peptide receptor 2

FSC: forward scatter

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GATA3: GATA binding protein 3

GC: germinal centre

G-CSF: granulocyte-colony stimulating factor

GM-CSF: granulocyte-macrophage-colony stimulating factor

GPCR: G protein-coupled receptor

H_2O_2: hydrogen peroxide

hBD: human β defensin 2

HBSS: Hank's balanced salt solution

hCAP18: human cationic antimicrobial protein

HDP: host defence peptide

HEV: high endothelial venule

HIF1α: hypoxia-inducible factor 1-alpha

HIV: human immunodeficiency virus

HKST: heat-killed *Salmonella typhimurium*

HLA: human leukocyte antigen

HNP1: human neutrophil peptide 1

HPLC: high performace liquid chromatography

i.p: intraperitoneal

IAV: influenza A virus

IBD: inflammatory bowel disease

Ig: immunoglobulin

IKBKE: inhibitor of nuclear factor κB kinase subunit ε

IKZF4: Ikaros family zinc finger 4

IL: interleukin

ILC: innate lymphoid cell

IMDM: iscove's modified dulbecco's media

IMQ: imiquimod

IRF: interferon regulatory factor

ITE: 2-(1' H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester

iTreg: induced regulatory T cell

KO: knockout

LN: lymph node

LPS: lipopolysaccharide

LTB4: leukotriene B4

MAC1: macrophage antigen 1

MAPK: mitogen-activated protein kinase

MCP: monocyte chemoattractant protein

CRAMP: cathelicidin-related antimicrobial peptide

MDSC: myeloid-derived suppressor cell

MHC: major histocompatibility complex

mins: minutes

mLN: mesenteric lymph node

MMP: matrix metalloproteinase

MOG: myelin oligodendrocyte glycoprotein

MPO: myeloperoxidase

MRI: magnetic resonance imaging

MS: multiple sclerosis

mTOR: mammalian target of rapamycin

NADPH: nicotinamide adenine dinucleotide phosphate

NET: neutrophil extracellular trap

NFATc1: nuclear factor of activated T cells, cytoplasmic 1

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NK: natural killer

ns: non-significant

nTreg: natural regulatory T cell

OVA: ovalbumin

P2X7: P2X purinoceptor 7

PAMP: pathogen-associated molecular pattern

PAF: platelet-activating factor

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PD1: programmed cell death protein 1

pDC: plasmacytoid dendritic cell

PDL1: programmed death ligand 1

PI: propidium iodide

PMN: polymorphonuclear neutrophils

PP: Peyer's patch

PP47: partial peptide 47

PRR: pathogen recognition receptor

PS: phosphatidylserine

RA: rheumatoid arthritis

ROCK: Rho-associated protein kinase

RNA: ribonucleic acid

ROR: RAR-related orphan receptor

ROS: reactive oxygen species

RPMI: Roswell Park Memorial Institute Medium

RSV: respiratory syncytial virus

RT-PCR: reverse transcription polymerase chain reaction

s.c.: subcutaneous

SCF: stem cell factor

SCS: subcapsular sinus

SDF: stromal-derived factor

SEA: *Schistosoma mansoni* soluble egg antigen

SIRPα: signal regulatory protein α

SOCS: suppressor of cytokine signalling

SSC: side scatter

STAT: signal transducer and activator of transcription

T-bet: T-box-containing protein expressed in T cells

Tc: cytotoxic T cell

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

TCF: transcription factor

TCR: T cell receptor

Tfh: follicular T helper cell

TGF: transforming growth factor

Th: T helper

TLR: Toll-like receptor

TMEV: theiler's murine encephalomyelitis virus

TNBS: trinitrobenzenesulfonic acid

TNF: tumour necrosis factor

TRAF6: tumor necrosis factor receptor-associated factor 6

Treg: regulatory T cell

UC: ulcerative colitis

UT: untreated

VDRE: vitamin D response element

VV: vaccina virus

WT: wildtype

XCL: chemokine (X-C motif) ligand

XCR: chemokine (X-C motif) receptor

x g: relative centrifugal force

CHAPTER 1

Introduction

The immune system is typically divided into two branches: innate (non-specific) and adaptive (acquired) immunity, both of which efficiently cooperate to protect the host from infection[1]. The innate immune system dates back to the first multicellular organisms and is the most evolutionary conserved[2]. It refers to non-specific physical, chemical and cellular defence mechanisms, which contribute to the generation of a rapid inflammatory response immediately after immune challenge[1,3]. These include physical barriers (e.g. skin, epithelial and mucosal linings of respiratory and gastrointestinal tracts), phagocytic cells (e.g. macrophages), the complement system and soluble proteins[1,3]. The innate immune system is activated following recognition of highly conserved molecular structures, or pathogen associated molecular patterns (PAMPs), by pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs)[1,4].

On the other hand, adaptive immunity refers to the more complex, antigen-specific immune response and can only be found in jawed fish and vertebrates[5]. The hallmarks of the adaptive immune system are the clonal expansion of B and T lymphocytes bearing antigen-specific receptors and the development of immunological memory[1,6]. Receptor specificity is generated by the somatic recombination and hypermutation of gene segments that encode B and T cell receptors[7]. B lymphocytes secrete antibodies that bind to antigens displayed by pathogens or extracellularly on infected cells, triggering the activation of mechanisms that attack and destroy them[1]. T lymphocytes help activate B cells, secrete cytokines and trigger programmed cell death pathways[1]. Unlike the innate immune system, adaptive immunity requires several days to mount an efficient response following its first pathogen encounter[1]. However, the effects of the adaptive immune system are long-lasting, highly specific and sustained by memory T and B cells, which allow for a more rapid and effective response to re-infection[8].

1.1 T Cells

1.1.1 T cell development

T cells are derived from haematopoietic stem cells that develop in the bone marrow and migrate to the thymus, where they undergo a series of maturation steps dependent on a

number of signalling events and molecular interactions with the local stromal cell compartment[9]. Receptor specificity is generated by somatic rearrangement of the gene loci encoding the T cell receptor (TCR) α, β, γ and δ chains, which give rise to either αβ or γδ lineages[7].

Double negative (DN) cells represent the earliest developing thymocytes; they lack the co-receptors CD4 and CD8, but can be subdivided based on their expression of CD44 and CD25[9]. $CD44^{-}CD25^{+}$ cells undergo β-selection, which selects cells that have successfully rearranged their TCR β chain locus[9]. Formation of a pre-TCR (pairing of the β chain with a surrogate α chain) promotes their survival and proliferation[9]. This induces the upregulation and expression of both CD4 and CD8[9]. These double positive (DP) cells then rearrange their TCR α chain to produce an $\alpha\beta$ TCR[9].

Positive selection of maturing T cells results in the survival of those capable of recognising and engaging with MHC-peptide complexes with appropriate affinity[10]. Conversely, negative selection ensures the elimination of thymocytes that bind self-peptide-MHC (major histocompatibility complex) complexes so strongly as to be potentially autoreactive[10]. The vast majority of developing T cells die by apoptosis during this process[9]. Following selection, the down-regulation of either co-receptor produces naïve $CD4^{+}$ or $CD8^{+}$ lymphocytes that exit the thymus and circulate the periphery[9].

Naïve T cells constantly circulate between secondary lymphoid organs via the blood and lymph in search of their cognate antigens[11]. However, they have also been shown to routinely traffic through non-lymphoid organs as well, including the liver, lungs and intestine[11,12]. Migration of T cells from the blood to these sites follows the general adhesion and signalling steps of cell trafficking: selectin-mediated rolling, chemokine-triggered activation and integrin-dependent arrest[13].

1.1.2 T cell activation

T cell activation results in the proliferation and differentiation of naïve lymphocytes, and is critical for the initiation and regulation of the immune response (**Figure 1.1**)[8]. Antigen-

presenting cells (APCs) such as dendritic cells (DCs), patrol the periphery to capture and process antigen[8]. Once captured, they then migrate to the lymphoid organs where they present antigen peptides in complex with MHC molecules to naïve T cells[8]. Interactions between the TCR, CD4/CD8 co-receptor and antigen-MHC complex results in the activation of downstream signalling pathways that promote T cell activation and differentiation[8].

In addition to antigen, a second co-stimulatory signal is also required to protect against premature or excessive T cell activation[8]. CD28 is expressed on all naïve T helper cells and its ligands, CD80 and CD86, are upregulated upon DC activation[14]. Furthermore, T cells that recognise antigen strongly specifically upregulate the expression of 4-1BB (CD137) and OX40[8,15]. Their respective ligands are typically found on APCs following pathogen recognition and provide survival signals to primed T cells[8,15]. These TCR and co-stimulatory signals induce robust interleukin-2 (IL-2) production and T cell proliferation[8].

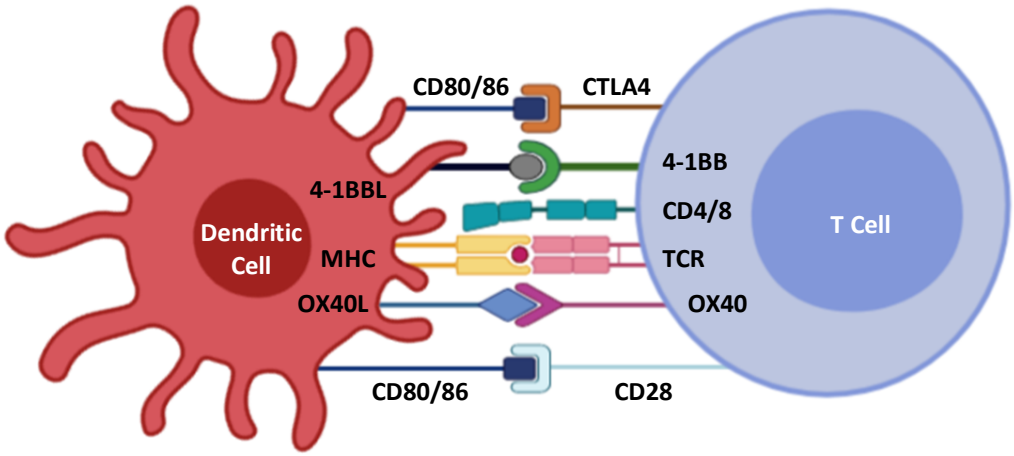


Figure 1.1: **T cell activation by a dendritic cell**. DCs present antigen peptides in complex with MHC molecules to naïve T cells. Interactions between the TCR, CD4/CD8 co-receptor and the antigen-MHC complex activate the T cell. A co-stimulatory signal mediated by CD28 is also required. CTLA4 is an inhibitory receptor that competes for CD28 and negatively regulates T cell activation. Binding of OX40 and 4-1BB to their respective ligands provide survival signals.

Antigen dose, the type of APC and time of differentiation have all been shown to contribute to the polarization of naive T cells[16]. For example, DCs are more potent inducers of IL-2 production and therefore stimulate the proliferation of T cells better than macrophages[17]. The cytokine environment is also critical for determining T cell lineage[8].

1.1.3 CD4⁺ T helper and CD8⁺ cytotoxic T cells

T lymphocytes can be broadly subdivided into CD4⁺ T helper cells and cytotoxic CD8⁺ T cells[1,8]. CD4⁺ T helper cells are essential for the development of adaptive immunity: they orchestrate a coordinated response from multiple other cell types via the production of diverse, pleiotropic soluble mediators[18]. For example, T helper cells play a key role in the maturation of B cells into antibody-producing plasma cells, as well as the recruitment of other immune leukocytes to sites of infection/inflammation[1,8]. CD4⁺ T cells recognise antigen peptides presented on MHC class II molecules by APCs and upon activation, can differentiate into several distinct subsets that produce different panels of cytokines[1,8].

Conversely, cytotoxic CD8⁺ T lymphocytes recognise antigen peptides presented by MHC class I molecules, which is expressed by all nucleated cells[1,8]. They are critical for immune defence against intracellular pathogens and tumour surveillance and employ multiple mechanisms to kill infected or malignant cells[1,19]. For instance, they produce TNFα and IFNγ, which exhibit anti-tumour and anti-viral activity respectively[20,21]. CD8⁺ T cells can also form an immunological synapse with infected cells, into which they release the contents of cytotoxic granules (perforin and granzymes)[19]. Perforin forms a pore in the membrane of the target cell, which allows serine proteases to enter and induce apoptosis[19,22]. Activated CD8⁺ T lymphocytes can also promote apoptosis via interactions between Fas ligand (FasL) and Fas[19]. Ligation of CD8⁺ FasL and target cell Fas induces the recruitment of the adaptor protein FADD (Fas-associated death domain) and binding of procaspase-8, resulting in the formation of the death-inducing signalling complex (DISC)[23]. This leads to the activation of effector caspase-3 by active caspase-8 and programmed cell death[23].

1.1.4 T helper cell subsets

In 1986, Coffman and Mossman suggested that different subpopulations of CD4⁺ T helper cells possessed distinct cytokine signatures that could differentially shape immune responses[24]. Two subsets were initially identified: Th1 and Th2[24]. Furthermore, they postulated that naïve T helper cells are not pre-determined to be either Th1 or Th2[24]. Instead, the environment in which they encounter antigen dictates their subsequent fate[24]. Since then, it has been established that the effector CD4⁺ T cell population is highly heterogenous[25].

A number of other T helper cell subsets have subsequently been identified based on the cytokines and transcription factors required for differentiation, the cytokines they produce, as well as their physiological function (**Figure 1.2**)[25].

Th1 lymphocytes are the primary producers of IFNγ and are critical for host immunity against replicating intracellular pathogens, such as *Salmonella typhimurium* and *Mycobacterium tuberculosis*[26]. Naïve $CD4^+$ T cells are driven to differentiate towards the Th1 lineage by IL-12 and IFNγ[27]. IFNγ activates the STAT1 (signal transducer and activator of transcription 1) signalling pathway and induces the expression of T-bet, the master transcription factor required for Th1 polarization[28]. IL-12 activates STAT4, which together with T-bet, promotes the production of IFNγ and suppresses Th2 development by downregulating GATA3[29,30]. Th1-derived IFNγ creates a positive feedback loop that promotes further Th1 responses, as well as activates and enhances the microbicidal activity of macrophages to combat infection[31].

Th2 cells are important for the eradication of helminths and extracellular parasites, but have also been implicated in the pathogenesis of asthma and allergy[26]. Th2 differentiation requires IL-4[32]. IL-4 activates STAT6, which upregulates the expression GATA3[33]. GATA3 is indispensable for Th2 development; differentiation of naïve T cells is diverted towards the Th1 lineage in GATA3-deficient mice[34]. GATA3 suppresses Th1 differentiation by downregulating STAT4 and inhibiting IFNγ, but together with STAT6, plays additional roles in the transcription of Th2-specific genes, including the type 2 cytokines IL-4, IL-5 and IL-13[35,36]. IL-4 and IL-13 promote B-cell proliferation and stimulate antibody production and class-switching to IgE[37]. IL-5 stimulates bone marrow production of eosinophils, as well as their activation and chemotaxis to affected tissues[38]. IL-13 enhances mucus production and is responsible for helminth expulsion and airway hypersensitivity[26].

The Th1/Th2 paradigm was re-evaluated and extended in light of the identification of the Th17 subset in 2005[39]. Th17 cells protect the host at mucosal surfaces against extracellular bacteria and fungi[26]. As their name suggests, Th17 lymphocytes produce large quantities of the pro-inflammatory cytokine IL-17, which has also been shown to drive immunopathology in various autoimmune conditions such as multiple sclerosis (MS), rheumatoid arthritis (RA) and psoriasis[39,40]. In mice, early commitment to the Th17 lineage requires TGFβ and IL-6,

which activates STAT3[41]. STAT3 upregulates IL-23R and the Th17 master transcriptional regulator, RORγt, which in turn induces the expression of IL-17[42,43]. Differentiation of human Th17 cells also requires IL-1β[44]. Despite minor differences in cytokine requirements, the molecular mechanisms responsible for inducing IL-17 production in human $CD4^+$ T cells are similar to those in mice.

Regulatory T cells (Tregs) are critical for maintaining immune homeostasis and promoting tolerance[26]. More specifically, Tregs secrete a number of immunomodulatory proteins (IL-10 and TGFβ) that downregulate the activity of other pro-inflammatory effector cells by suppressing their activation, proliferation and cytokine production[26]. For example, Treg-derived IL-10 inhibits intestinal inflammation in a T cell transfer model of colitis[45]. Tregs constitutively express CD25 (IL-2Rα chain) and CTLA4, which serve both as markers for this subset, as well their regulatory function[46,47]. Natural Tregs (nTreg) develop in the thymus and require the engagement of CD28 and expression of the transcription factor FOXP3 (Forkhead Box P3) for their development[26,48]. However, strong TCR signalling, suboptimal co-stimulation and high concentrations of TGFβ have been shown to induce the expression of FOXP3 in naïve T cells *in vitro*, as well as in the periphery[49,50]. These induced Tregs (iTreg) are phenotypically and functionally similar to thymic-derived nTreg, in that they are anergic, suppressive and capable of inhibiting disease *in vivo*: Chen et al. demonstrated that TGFβ-induced Tregs prevented the development of pathogenesis in a mouse model of allergic asthma, as well as suppressed antigen-specific $CD4^+$ T cell proliferation in an ovalbumin (OVA) peptide TCR transgenic adoptive transfer model[49].

Several other T helper cell subsets have been identified, including Th9, Th22 and T follicular helper (Tfh) cells[26]. Th9 lymphocytes are one of the most recently discovered and secrete large quantities of IL-9, a growth factor historically associated with the Th2 phenotype[51]. Differentiated Th2 lymphocytes can undergo Th9 polarization when stimulated with TGFβ, however, naïve $CD4^+$ T cells can also be driven towards the Th9 lineage by TGFβ and IL-4[52]. Th22 lymphocytes produce IL-22, which acts on non-haematopoietic cells such as epithelial cells and keratinocytes to promote wound healing and maintain barrier integrity[53,54]. The aryl hydrocarbon receptor (AHR) drives Th22 differentiation, but the polarizing cytokines required by this lineage remain unknown[53]. Finally, T follicular helper (Tfh) cells can be found

in the secondary lymphoid organs and play essential roles in the formation of germinal centres and B cell antibody production[55]. Tfh lymphocytes require IL-21 and IL-6 for their differentiation, which induces the expression of the Bcl6 (B Cell Lymphoma 6) transcription factor[56].

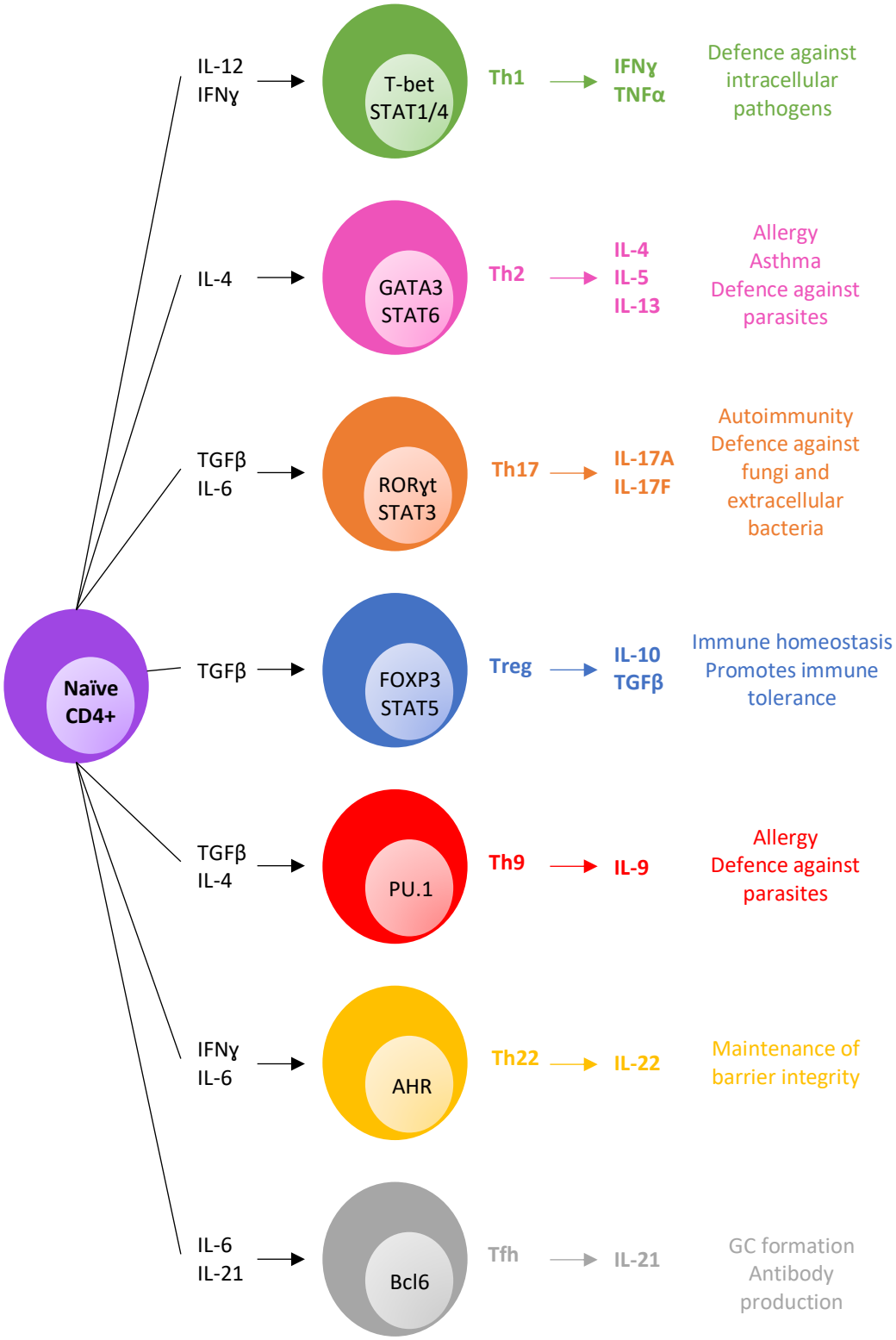


Figure 1.2: **CD4⁺ T helper and regulatory subsets**. Naive CD4⁺ T cells can differentiate into distinct subsets depending on the cytokine environment. These are characterized by their expression of lineage-specific transcriptional regulators, as well as the secretion of key cytokines. GC: germinal centre

1.2 Th17 Cells

1.2.1 Th17 differentiation

Th17 polarization can be split into three phases: 1) differentiation, mediated by IL-6 and/or TGFβ and IL-1β 2) IL-21-induced self-amplification, 3) and stabilisation, which is driven by IL-23 (**Figure 1.3**)[26].

IL-6 plays an indispensable role in initiating Th17 differentiation by activating STAT3, which drives the transcription of RORγt[26]. Humans with STAT3 mutations have lower numbers of IL-17-producing T cells and as a result, are prone to recurrent opportunistic pulmonary infections[57]. Furthermore, STAT3 ablation in $CD4^+$ T cells results in increased expression of T-bet and FOXP3, as well as protection against several autoimmune pathologies, such as EAE (experimental autoimmune encephalomyelitis, the mouse model of MS)[58,59]. In addition to driving RORγt expression, STAT3 can also bind to the IL-17A and IL-17F promoters, thereby directly promoting their expression[60].

RORγt is the master regulator of Th17 differentiation: ectopic expression of *RORC2* in primary human T cells induces a cytokine and chemokine receptor profile consistent with bona fide human Th17 cells[61]. Unlike STAT3, RORγt-deficiency leads to restricted, but not complete interruption, of Th17 cytokine expression[62].

TGFβ is also critical for Th17 development: mice over-expressing TGFβ generate more Th17 cells, whereas those with defective TGFβ signalling display profoundly impaired Th17 differentiation *in vivo*[63,64]. TGFβ plays an important but indirect role by suppressing T-bet and GATA3, thereby inhibiting $CD4^+$ T cells from adopting alternate Th1 or Th2 cell fates[65]. Moreover, one study has shown that TGFβ inhibits SOCS3, a negative regulator of STAT3 signalling and therefore Th17 differentiation[66]. However, this cytokine also plays critical roles in Treg polarization[67]. More specifically, TGFβ alone and at high concentrations induces FOXP3 expression, whereas low concentrations of TGFβ and the presence of IL-6 enhance STAT3 activation and upregulates RORγt[67].

There has been some debate as to the absolute requirement for TGFβ during human Th17 development. For example, one group has shown that TGFβ inhibited IL-17 production in memory T cells when added alone or in combination with another Th17-polarizing cytokine (IL-1β, IL-23 or IL-6)[68]. Furthermore, Chen et al. demonstrated that naïve human T cells failed to differentiate in response to TGFβ and IL-6, despite the fact that they promoted RORγt expression[69]. Some studies suggest that IL-1β can substitute the effect of TGF-β in driving Th17 differentiation: Acosta Rodriguez and colleagues demonstrated that IL-1β and IL-6 were essential for inducing IL-17 production in cell-sorted naïve human T cells[70]. However, Manel et al. found that TGFβ, together with IL-23 and IL-1β induced human Th17 differentiation under serum-free conditions[71]. They postulated that culture medium contained serum-derived TGFβ or AHR ligands, which was responsible for previously inaccurate findings that led some to conclude that this cytokine was not required[71].

The second, self-amplification phase of Th17 differentiation is crucial for mounting a robust immune response[26]. This is driven by IL-21, which is produced in significant amounts by the Th17 cells themselves[72]. Unlike IFNγ and IL-4, which are capable of creating positive feedback loops during Th1 and Th2 differentiation respectively, IL-17 does not amplify Th17 polarization[73]. This is because IL-17 is neither a growth nor a differentiation factor, and its receptor is not expressed on T cells[73]. IL-21 is induced by IL-6 in activated T cells in a STAT3-dependent process and has been shown to suppress FOXP3 expression and inhibit IFNγ production in naïve $CD4^+$ T cells, thereby promoting Th17 development whilst simultaneously antagonising differentiation of other cell lineages[72]. IL-21 deficiency impairs Th17 differentiation and protects against EAE[72]. However, some studies suggest that IL-21 is not an absolute requirement for Th17 development: Sondereggar and colleagues demonstrated that the differentiation of IL-17-producing $CD4^+$ T cells, their recruitment to inflamed organs and the development of autoimmune disease was not affected in *Il21R*$^{-/-}$ and *Il21*$^{-/-}$ mice[74].

The final stage of Th17 development is driven by IL-23, which is produced predominantly by activated APCs in response to exogenous or endogenous stress signals[26]. IL-23 is unable to induce Th17 differentiation on its own; naïve $CD4^+$ T cells are not responsive to IL-23 because they do not express the IL-23 receptor (IL-23R), which is induced by IL-6 and IL-21[75]. However, several reports have shown that IL-23 synergizes with other Th17 differentiation factors to

stabilize the population by activating or maintaining effector function genes (*Rorc*, *Il17* and *Il23r),* whilst repressing those that destabilize the Th17 lineage (*Il2, Il27*)[76]. Indeed, IL-23R-deficient Th17 cells become arrested at an early activation stage, are unable to downregulate IL-2 and fail to maintain their IL-17 expression[77]. In addition, Sun and colleagues recently demonstrated that RORγt also contributes to the maintenance of pathogenicity of Th17 cells by suppressing IL-10 production[78].

Several other transcription factors have been implicated in Th17 development[26]. RORα is induced by IL-6/TGFβ, and together with RORγt, synergistically enhances Th17 differentiation by promoting the expression of IL-17[62]. It has been suggested that the residual IL-17 production observed in RORγt-deficient cells is dependent on the activity of RORα[62]. Interestingly, mice deficient for both RORα and RORγt show complete inhibition of Th17 differentiation[62].

AHR, a ligand-activated transcription factor that integrates environmental, dietary , microbial and metabolic cues, has also been shown to promote Th17 polarization[26]. Treatment of mouse $CD4^+$ T cells cultured under Th17-driving conditions with AHR ligands such as FICZ, increased the percentage of $IL\text{-}17^+$ lymphocytes and enhanced autoimmune pathology during EAE[79]. Furthermore, Veldhoen et al. demonstrated that culturing T cells in Iscove's modified Dulbecco's medium (IMDM), which is richer in aromatic amino acids that give rise to natural AHR agonists, resulted in higher Th17 expansion compared to RPMI[80]. However, loss of AHR does not cause complete abortion of Th17 differentiation, although this has been associated with a loss of IL-22 expression[79]. Kimura and colleagues suggested that AHR acts indirectly to inhibit the activity of STAT1 by acting as an E3 ubiquitin ligase, targeting it for degradation and consequently suppressing Th1 development[81].

BATF is also thought to play an important role during Th17 polarization: T cells from BATF knockout mice are incapable of inducing the expression of RORγt and IL-21 and are resistant to EAE[82]. Similarly, IRF4-deficiency in $CD4^+$ T helper cells leads to the downregulation of IL-23R and RORγt, which characterises a profound intrinsic defect in IL-17 production and in the autocrine IL-21 loop[83]. Mudter and colleagues demonstrated that IRF4 directly binds to the IL-17 promoter and induces the mucosal expression of this cytokine, which subsequently drives Th17-dependent colitis[84].

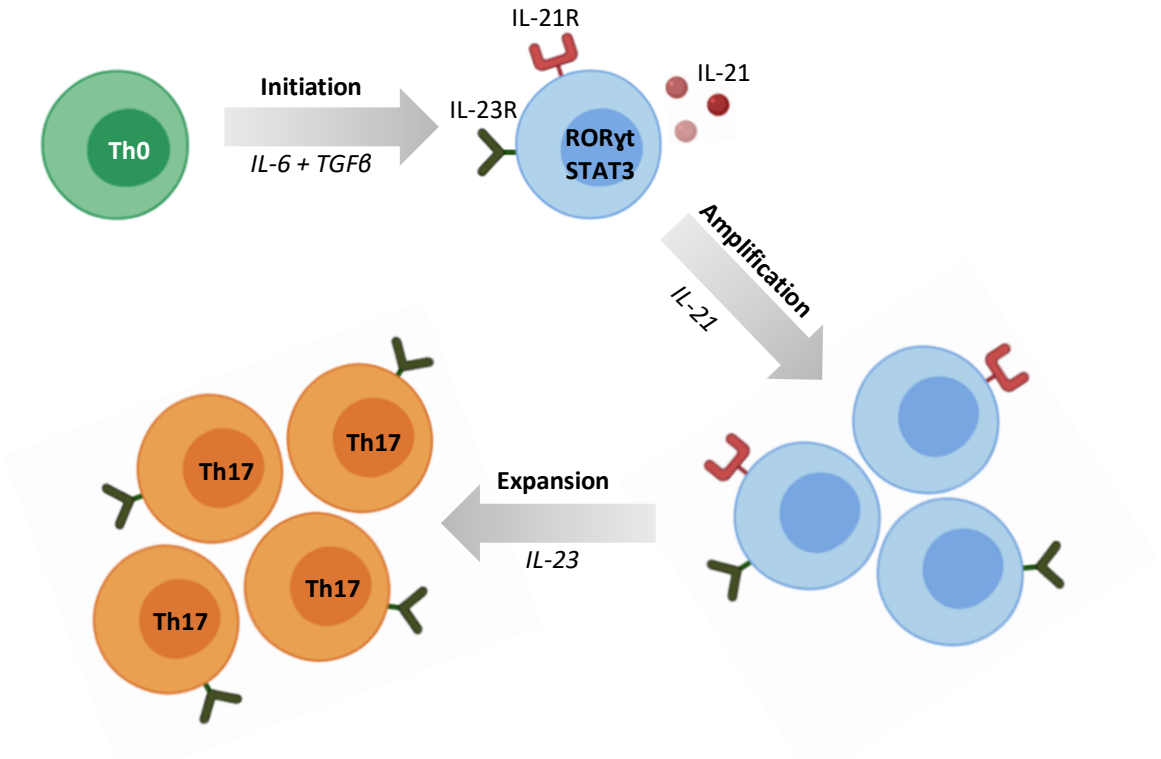


Figure 1.3: **Mouse Th17 differentiation**. Th17 polarization in mice can be split into three phases. Differentiation is mediated by IL-6 and TGFβ, which activate STAT3 and induce the expression of RORγt. The self-amplification phase is driven by IL-21. IL-23 is responsible for the expansion and stabilisation of the Th17 phenotype.

1.2.2 Negative regulation of Th17 differentiation

Numerous factors associated with the development of other T helper cell subsets are potent inhibitors of Th17 differentiation. IFNγ increases T-bet expression, which subsequently prevents IL-23-mediated expansion of Th17 lymphocytes in the intestine[85]. IL-27 is another classical Th1-associated cytokine produced by macrophages and DCs, that blocks the initial stages of Th17 differentiation by directly suppressing RORγt and RORα in a STAT1-dependent manner[86]. However, committed IL-23R^{+} Th17 cells downregulate IL-27R and are therefore resistant to IL-27-mediated suppression[87]. In addition, IL-2 signalling induces STAT5, which can displace STAT3 from the IL-17 promoter and subsequently repress its transcription[88]. Cytokines associated with the Th2 lineage also inhibit Th17 polarization. For instance, IL-4 selectively suppresses the transcription and secretion of Th17-polarizing cytokines such as IL-23 by cells of the innate immune system[89].

1.2.3 Microbiome-dependent regulation of Th17 cells

During homeostasis, Th17 cells are predominantly located at barrier sites, such as in the skin and gastrointestinal tract[43]. The frequency of these barrier-resident Th17 lymphocytes is dramatically reduced in germ-free (GF) mice, suggesting that their development is dependent on commensal bacterial colonization[90]. Specific species have been shown to drive Th17 differentiation[90]. For example, Ivanov et al. established that segmented filamentous bacteria (SFB) are sufficient to reconstitute the Th17 population, which homed to the lamina propria of both the small and large intestines in GF mice following colonization[91]. SFB are Gram-positive, spore-forming microorganisms that are closely related to *Clostridia* and *Bacteroides* species[91]. SFB induce the upregulation of several genes in epithelial cells, including serum amyloid antigen (SAA), which promotes the development of Th17 lymphocytes by promoting the production of IL-1β by dendritic cells[92,93]. Commensal dysbiosis and decreased levels of SFB have been observed in mice fed a high-fat diet, which correlates with a reduced number of Th17 cells[92,94]. Furthermore, SFB-induced Th17 lymphocytes were shown to exacerbate disease in a mouse model of arthritis[92,95]. However, SFB can only be detected in humans during the first 36 months after birth and as such, the role this bacterial species plays in inducing the generation of intestinal Th17 cells is controversial[92].

Adenosine 5'-triphosphate (ATP)-producing bacteria have also been shown to promote the accumulation of Th17 cells within the lamina propria[96]. Atarashi and colleagues demonstrated that systemic or rectal administration of ATP into GF mice activated a unique subset of $CD70^{high}$ $CD11c^{low}$ cells that enhanced Th17 differentiation, resulting in the exacerbation of T cell-mediated colitis[97].

1.2.4 Th17-related cytokines

1.2.4.1 Interleukin-17

As their name suggests, Th17 cells produce IL-17[39]. IL-17 is part of a family of six related cytokines (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17-F), all of which possess conserved residues in their C-terminal region[98]. $CD4^+$ T cells can express IL-17A and IL-17F, which are

highly homologous and can form three different disulphide-linked dimers (IL-17F/F, IL-17A/A, IL-17F/A)[98]. All three dimeric forms of IL-17 trigger qualitatively similar signalling pathways[98]. However, IL-17A homodimers are considered more potent than IL-17F[99].

The IL-17 receptor family consists of five members (IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE)[100]. IL-17A and IL-17F signal through a heteromeric receptor complex consisting of the IL-17RA and IL-17RC chains, both of which are single transmembrane proteins and ubiquitously expressed in various cell types[100]. The absence of either chain has been shown to prevent IL-17-driven pro-inflammatory cytokine secretion[101]. Other receptors including IL-17RB and IL-17RE have been identified as specific receptors for IL-17B and IL-17C, respectively[100].

Binding of IL-17 to its receptor induces the activation of the adaptor protein ACT1, which is required for the recruitment of TNFR-associated factor 6 (TRAF6)[100]. TRAF6 is an essential upstream activator of the canonical nuclear factor–κB (NFκB) and mitogen-activated protein kinase (MAPK) intracellular pathways[100]. Collectively, these factors trigger the stabilization and transcriptional induction of IL-17-induced genes (**Figure 1.4**)[100].

IL-17 has pleiotropic effects and is an important mediator in tissue inflammation. Its main effect is the recruitment and activation of neutrophils[102]. For instance, it has been shown to increase neutrophil survival by directly inhibiting apoptosis in inflamed tissues[103]. Laan and colleagues demonstrated that subcutaneous injection of IL-17A into mice accelerates neutrophil development[102]. More specifically, IL-17A promotes granulopoiesis by inducing the secretion of granulocyte-colony stimulating factor (G-CSF) in bone marrow stromal cells, which synergizes with the transmembrane form of stem cell factor (SCF) to induce the differentiation of $CD34^+$ progenitors into neutrophil precursors *in vitro*[104,105]. In addition, IL-17 induces the expression of chemokines such as CXC-chemokine ligand 1 (CXCL1), CXCL2 and CXCL5, all of which are neutrophil chemoattractants and promote neutrophil recruitment[98]. Both neutrophil recruitment and granulopoiesis are impaired in *Il17ra*$^{-/-}$ mice, rendering them susceptible to microbial infection[106]. Furthermore, Liang et al. showed that neutralization of IL-17A homodimers strongly blocked neutrophilic airway inflammation

induced by the adoptive transfer of ovalbumin (OVA)-specific Th17 lymphocytes and subsequent airway challenge with antigen[107].

Various other cytokines and inflammatory mediators are induced by IL-17, including IL-6, IL-1β and antimicrobial peptides (AMPs)[98]. For example, IL-17 enhances the expression of β-defensin 2 and S100A9 in primary keratinocytes[108].

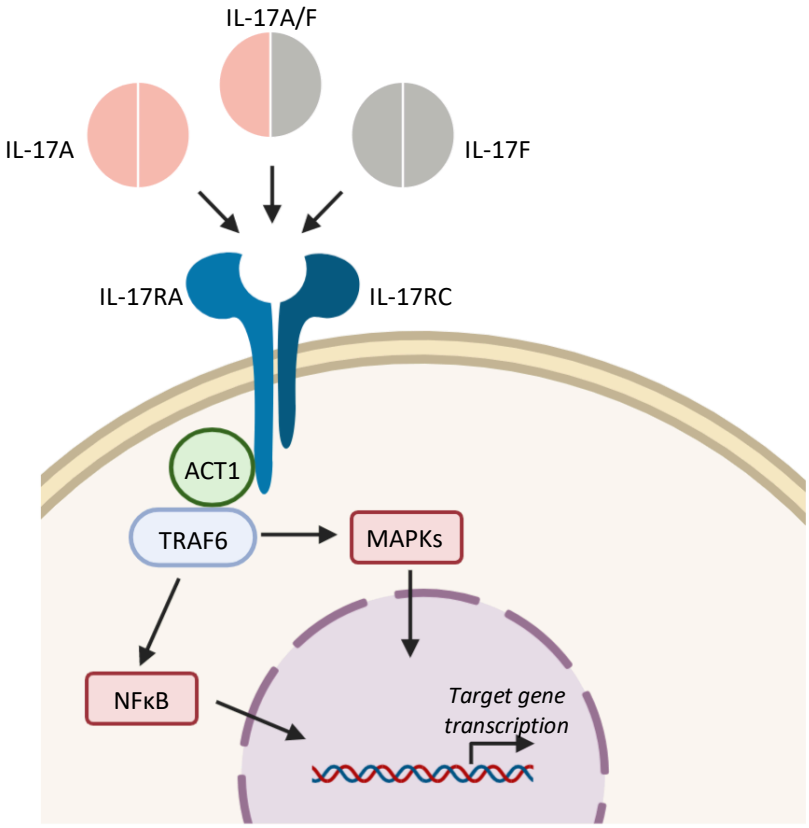


Figure 1.4: Simplified IL-17 signalling. IL-17A and IL-17F dimers, as well as IL-17A/F heterodimers bind to the IL-17 receptor complex, composed of IL-17RA and IL-17RC chains. Receptor signaling activates ACT1, which recruits TRAF6. This results in the activation of the NFκB and MAPK signalling pathways that induce the transcription of target genes.

1.2.4.2 Other Th17-related cytokines

Mature Th17 cells produce several other cytokines besides IL-17. For instance, the encephalitogenicity of Th17 cells has been shown to be dependent on IL-1β- and IL-23-induced production of granulocyte-macrophage-colony stimulating factor (GM-CSF)[109]. Th17-derived GM-CSF induces the activation, maturation and differentiation of pro-inflammatory myeloid cells, which contribute to demyelination in EAE[109]. GM-CSF is also essential for the secretion of Th17-stimulating cytokines, such as IL-23 and IL-6, by dendritic cells and macrophages, thereby further amplifying IL-17-driven pathology[110].

Th17 cells also produce IL-22, a member of the IL-10 family of cytokines[108]. IL-21 triggers IL-22 production in $CD4^+$ T cells by activating STAT3, which controls the epigenetic status of the *Il22* promoter and its interaction with AHR[111]. Conversely, the transcriptional repressor c-Maf can also bind to the *Il22* promoter and mediate TGFβ-dependent suppression of IL-22 production in Th17 cells[112]. The IL-22 receptor is a heterodimer composed of the IL-10R2 and IL-22R1 subunits and is almost exclusively expressed on cells of non-haematopoietic origin[113]. IL-22 provides tissue-protective effects and is primarily involved in the preservation of mucosal barriers[113]. For instance, IL-22 exhibits anti-apoptotic properties that, together with its capability to promote cellular proliferation, aid in wound healing and tissue repair[114]. Yeste et al. demonstrated that IL-21 and AHR signalling limit mucosal inflammation during DSS-induced colitis through the production of IL-22[111]. IL-22 can also promote the expression of several antimicrobial peptides at mucosal surfaces to protect the host from microbial infections[108]. Aujla and colleagues observed that IL-22-knockout mice displayed increased mortality in response to infection with *Klebsiella pneumoniae* and that this was due to increased bacterial adhesion and invasion[115]. However, IL-22 can be pathogenic in settings of chronic inflammation; increased proliferation and cell survival can lead to epidermal hyperplasia[116].

1.2.5 Th17 Cells in health and disease

1.2.5.1 Th17 cells in bacterial infection

The Th1 lineage is typically considered the main defence against intracellular pathogens[26]. However, Th17 lymphocytes are critical for the recruitment and early activation of neutrophils and therefore play important roles in host protection against numerous bacterial species[117]. For instance, TLR4 expression in the lungs following *K. pneumoniae* infection promotes IL-23 production by DCs, leading to the release of IL-17 from both $CD4^+$ and $CD8^+$ T cells and subsequent neutrophil chemotaxis[118]. Furthermore, IL-17RA-deficient mice display impaired protective responses against *Streptococcus pneumoniae,* which is associated with subdued pneumococcal killing by polymorphonuclear cells[119]. Similarly, during *M. tuberculosis* infection, IL-17A promotes the formation of mature granulomas and induces the expression of CXCL9, CXCL10 and CXCL11, which recruit IFNγ-producing cells and promote the development of a protective Th1 response[120].

On the other hand, Th17 cells can also promote inflammation and subsequently induce harmful tissue damage during bacterial infection[117]. For example, Chung et al. demonstrated that the formation of abscesses that contribute to morbidity following intraperitoneal injection of *Staphylococcus aureus* or *Bacteroides fragilis* is dependent on Th17-derived IL-17[121].

1.2.5.2 Th17 cells in viral infection

Th17 cells are generally considered detrimental to the host during viral infection[122]. Hypercytokinemia of specific chemokines and cytokines has been associated with severe and often fatal cases of human influenza infection (e.g. H5N1)[123]. Bermejo-Martin and colleagues demonstrated that severe disease with respiratory involvement following infection with nvH1N1, a pandemic variant of the influenza virus, is characterized by early secretion of Th17 (and Th1) cytokines[123]. Furthermore, IL-17 suppresses the Th1 response by preventing Th1 differentiation and inhibiting the production of IL-2 and IFNγ, both of which induce cytotoxic T lymphocyte activity and anti-viral functions[122]. For example, Hou et al. demonstrated that Th17 cells enhance the persistence of Theiler's murine encephalomyelitis virus (TMEV) by

inhibiting target cell destruction by cytotoxic T lymphocytes[124]. In addition, Th17 cells were found to inhibit type 1 immunity and drive immunopathology in the liver during murine hepatitis[125].

However, IL-17$^+$ lymphocytes also play beneficial, if indirect roles, during viral infection. For instance, CD8$^+$ IL-17$^+$ (Tc17) cells induce an influx of neutrophils that protect the host after lethal influenza challenge[126].

1.2.5.3 Th17 cells in fungal infection

Th17 cells play an important role in host defence against fungal pathogens[98]. For instance, IL-17 is essential for protective immunity against *Candida albicans*, a commensal microorganism of the human oral cavity, gastrointestinal tract and reproductive mucosa[127]. Genetic polymorphisms leading to defects in Th17 differentiation and proliferation or IL-17 receptor signalling are associated with chronic mucocutaneous *Candida* infections[127]. For example, this occurs with autosomal recessive deficiency of the IL-17RA receptor[128]. IL-17 acts on epithelial and mesenchymal cells to upregulate the expression of pro-inflammatory cytokines such as IL-6, the neutrophil-recruiting chemokines CXCL1 and CXCL5, as well as antimicrobial peptides, all of which limit fungal growth and burden[127,129]. Conti and colleagues demonstrated that Th17-deficient and IL-17R-deficient mice experienced severe oropharyngeal candidiasis compared to Th1-deficient animals, which was associated with impaired neutrophil recruitment and AMP (S100A8, S100A9 and β-defensin 3) production[129].

1.2.5.4 Th17 cells in autoimmune disease

IL-17-secreting cells have been implicated in a number of autoimmune and chronic inflammatory conditions[98]. Psoriasis is a disease of the skin, characterized by epidermal hyperplasia due to abnormal differentiation of basal keratinocytes and leukocyte infiltration[130]. The expression of classical Th17-related genes, such as *RORC*, *IL17A* and *IL22* is upregulated in patients with psoriasis compared to healthy controls[130]. Psoriatic epidermal Th17 cells produce IL-17 and IL-22, which directly stimulate keratinocyte proliferation and inhibit epidermal differentiation, respectively[131,132]. Furthermore, DC-derived IL-23 has been

shown to drive psoriatic plaque formation in mice via activation of innate IL-17/IL-22–producing lymphocytes[133]. IL-17 also helps create a chronic inflammatory environment by inducing the release of neutrophilic chemoattractants by keratinocytes, which promotes granulocytic skin infiltration[134]. Interestingly, Chiricozzi et al. identified a complex interplay between IL-17 and TNF-α, in which they synergistically contributed to many of the key inflammatory pathways in psoriasis[135].

Th17 cells have been shown to play a critical role in driving pathology in rheumatoid arthritis (RA)[136]. For example, Nakae et al. showed that IL-17-deficient mice are resistant to collagen-induced arthritis and that IL-17 was responsible for the priming of collagen-specific T cells and collagen-specific IgG2a antibody production[137]. Furthermore, human patients with early inflammatory arthritis display enhanced and persistent levels of IL-17 in the peripheral blood, as well as increased numbers of Th17 cells compared to healthy human controls, which positively correlate with increased disease activity[138]. IL-17 stimulates the recruitment of neutrophils and monocytes, which in turn promotes joint inflammation and articular cartilage breakdown[136,139]. For instance, IL-17 is chemotactic for monocytes at concentrations typically detected in RA synovial fluid and Lubberts et al. determined that the ectopic expression of IL-17 intra-articularly enhanced neutrophil migration into the joints of mice[140,141]. Moreover, several studies have shown that IL-17 plays a major role in amplifying the effects of macrophage-derived cytokines[140]. IL-17 enhances TNFα-induced synthesis of IL-1β ,IL-6, and IL-8 in skin and synovial fibroblasts, thereby promoting inflammation[142].

There is a strong association between multiple sclerosis (MS), Th17 cells and IL-17[143]. IL-17 knockout mice show delayed onset, decreased severity scores and early recovery of EAE (the mouse model of MS)[144]. Furthermore, IL-23-deficient animals are resistant to EAE induction: Th17 cells from these mice cannot infiltrate the CNS and remain in the lymph nodes[77]. In humans, high levels of IL-17 can be detected in the lesions and CSF of MS patients[143]. Hedegaard and colleagues suggested that autoantigen-driven Th17 proliferation and levels of IL-17 were associated with disease activity, as determined by the number of active plaques detectable by magnetic resonance imaging (MRI)[145]. It has been postulated that Th17 cells play a more relevant role in the initial phases of EAE and MS, by mediating the breakdown of the blood brain barrier (BBB) and the recruitment of other pathogenic immune cells[143]. IL-17

promotes the formation of reactive oxygen species (ROS) within endothelial cells, enhances the activation of matrix metalloproteinase-3 (MMP-3), attracts neutrophils and induces the production of other pro-inflammatory cytokines such as IL-1β and TNFα[146]. This results in the impaired integrity of the BBB, the recruitment of large numbers of neutrophils, monocytes and macrophages, sustained myelin and axonal damage and neuronal apoptosis[146]. In addition, ROS generation leads to the upregulation of endothelial adhesion molecules and increased transmigration of inflammatory cells via the BBB, thereby allowing easier access of myelin-specific T cells from systemic circulation to a usually immune-privileged site[147].

Th17 cells play an important role in inflammatory bowel disease (IBD)[148]. Multiple genome-wide association studies have demonstrated that polymorphisms within several Th17-related genes are associated with IBD, including *IL23R* and *STAT3*[148]. The gastrointestinal mucosa of patients with Crohn's disease (CD) and ulcerative colitis (UC) is infiltrated with high numbers of Th17 cells[149]. In line with this, tissue biopsies from inflamed colons show elevated levels of Th17-associated cytokines compared to healthy controls[149]. Raza and Shata found that the levels of IL-17 secreted by PBMCs from UC patients correlated with disease activity[150]. IL-17 has been shown to trigger and amplify inflammation, by stimulating colonic epithelial cells to produce pro-inflammatory cytokines and chemokines such as IL-6, IL-8 and MCP-1 (monocyte chemoattractant protein) via NF-κB and MAPK pathways[151]. This is supported by the fact that IL-17R knockout mice are protected from TNBS-induced colitis[152]. However, IL-17 has also been reported to have tissue-protective effects in the gut: Ogawa et al. demonstrated that IL-17 neutralization lead to exacerbated intestinal inflammation in response to dextran sodium sulphate (DSS)[153]. Similarly, Th17-derived IL-22 has also been shown to play a protective role during IBD[148]. For example, IL-22-deficency is associated with worsening inflammation in mouse models of DSS-induced acute and chronic colitis[154]. The local protective effects of IL-17 and IL-22 are mediated by the induction of mucin production and the restoration of goblet cells, thereby increasing mucosal barrier function[148].

1.3 Neutrophils

Neutrophils are the most abundant innate immune cell in the human peripheral blood and comprise 60-70% of all leukocytes[155]. Previously, these short-lived effector cells were thought to have a half-life of less than a day (1.5 hours in mice, 8 hours in humans)[156]. However, a recent study by Pillay and colleagues demonstrated that neutrophils can have a circulatory lifespan of up to 12.5 hours in mice and an incredible 5.4 days in humans[157]. However, the methodological approach employed almost certainly also labelled bone marrow neutrophils and it is therefore possible that this is a considerable overestimate[158]. Nonetheless, neutrophil longevity increases several fold during inflammation and after migrating to the tissues, where they receive enhanced survival signals in the form of various cytokines, growth factors and bacterial products[156].

1.3.1 Neutrophil development

Neutrophils are produced in the bone marrow from hematopoietic stem cells[159]. In humans, around 10^{11} neutrophils are produced each day[155]. Common myeloid progenitors (CMPs) undergo a series of transformations in a highly regulated process that is mediated by different combinations of transcription factors expressed at specific stages of neutrophil development (**Figure 1.5**)[159]. The first neutrophil-committed progenitor, the promyelocyte, arises from the myeloblast state[159]. Promyelocytes are capable of dividing and can be recognised by their round nucleus and dark cytoplasm[160]. Myelocytes represent the last cell in the mitotic pool; the inhibition of proliferative genes and the upregulation of anti-proliferative factors induces the generation of metamyelocytes, marking the beginning of terminal differentiation[159]. These can be identified by their kidney-shaped nucleus and clear cytoplasm[160]. Banded neutrophils, in which the nucleus adopts a horseshoe shape, are the penultimate developmental stage[160]. These immature cells cannot be found in the peripheral blood during homeostasis but have been identified in the circulation during periods of acute infection or inflammation[160].

Mature neutrophils contain granules and secretory vesicles that store specific proteins relevant to their function, including antimicrobial peptides, proteases, membrane-bound receptors and extracellular matrix proteins to name but a few[161]. Neutrophil granule

formation is hierarchical and their distinct composition is dependent upon the timing at which they are formed, as opposed to protein sorting (targeting-by-timing hypothesis) (**Figure 1.5**)[162].

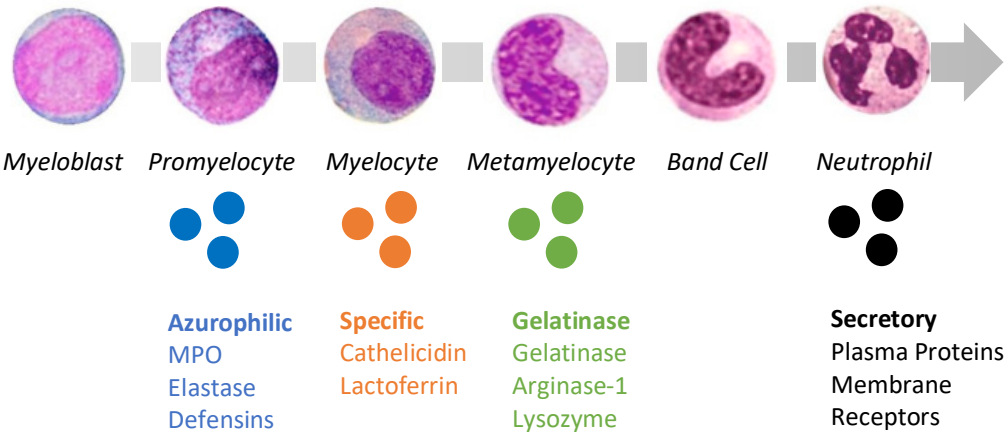


Figure 1.5: **Neutrophil development**. Modified from Brianna et al., 2019. Neutrophil progenitors undergo a series of transformations which coincide with granule formation. Their distinct composition is dependent upon the timing at which they are formed.

Azurophilic (primary) granules develop in myeloblasts and early promyelocytes[159]. These early-appearing granules were originally defined by their high myeloperoxidase (MPO) content, which accounts for almost 5% of the neutrophil's total dry weight[159]. However, they also contain a number of acidic hydrolases (elastase) and antimicrobial proteins (defensins)[159]. Secondary, or specific, granules are produced in myelocytes and are rich in antibiotic substances, including cathelicidin and lactoferrin[159]. Gelatinase (or tertiary) granules form during the transition of metamyelocytes to banded neutrophils[159]. As their name suggests, this subset possess high levels of gelatinase, as well as matrix metalloproteinases, which are important for the degradation of the vascular basement membrane and neutrophil extravasation into inflamed tissues[159]. Secretory vesicles can only be found in mature, segmented cells[159]. They contain an important reservoir of membrane-associated receptors, including CD10, CD11b and CD16, which are required for firm adhesion to the activated endothelium and migration into tissues[159]. The newly-discovered ficolin-1 granules are enriched for the microbial lectin, ficolin-1, and constitute the fourth and final subset[159]. Ficolin-1 is a very complex protein that is synthesized during the transition from

myelocytes to metamyelocytes, but localizes in granules that form in segmented cells during the final stages of neutrophil development[159]. The exact role of these ficolin-1 granules remains to be determined but it is possible that they provide rapid release of pattern recognition molecules that activate the lectin complement pathway[163].

1.3.2 Neutrophil recruitment

Fully mature neutrophils exhibit low expression of CXCR4 and increased CXCR2[164]. CXCR4 promotes the retention of neutrophils in the bone marrow where bone marrow stromal cells produce its ligand, CXCL12[165]. Mice with a myeloid lineage-restricted deletion of CXCR4 display neutrophilia in the peripheral blood[166]. The downregulation of this receptor therefore allows the release of mature neutrophils into circulation[164]. G-CSF also promotes neutrophil exit from the bone marrow by upregulating CXCR2 ligands (e.g. CXCL2) expressed by endothelial cells and downregulating CXCL12 in the bone marrow[165].

Circulating neutrophils are recruited from the blood and mobilized to sites of infection or inflammation through the leukocyte adhesion cascade, which consists of the following steps: tethering, rolling, adhesion, crawling and transmigration[156]. More specifically, direct activation of pattern-recognition receptors and the release of inflammatory mediators from tissue-resident sentinels induce the upregulation of adhesion molecules by endothelial cells[156]. These include P- and E-selectins, which bind to their glycosylated ligands with relatively low affinity, promoting the tethering and rolling of free-flowing neutrophils[156]. The activated and inflamed endothelium is also decorated with chemokines; activation of neutrophil G-protein-coupled chemokine receptors stimulates conformational unfolding of β1 and β2 integrins (e.g. MAC-1, LFA-1), which increases the affinity for their ligands (ICAM-1) and mediates firm adhesion[156]. Neutrophils finally leave the vasculature and gain access to the peripheral tissues via transmigration, which requires the release of specific proteases that possess enzymatic activity against the extracellular matrix (e.g. MMP9)[156]. Once extravasated, neutrophils follow chemotactic gradients to reach the site of inflammation and complete their functions[156].

Neutrophils can also be recruited to the lymph nodes via the afferent lymphatics or by exiting the circulation through high endothelial venules (HEVs)[167–169]. For instance, Hampton and colleagues demonstrated that neutrophils re-localized to the subcapsular sinus of draining lymph nodes following bacterial injury, indicating they entered via the lymphatics[168]. This was mediated by CD11b and CXCR4 and induced lymphocyte proliferation[168]. Similarly, HEVs are the major route of entry of blood-borne neutrophils into tumour-draining lymph nodes, which is dependent on interactions between neutrophil L-selectin/CXCR2 with addressin/CXCL2[167]. Beauvillain et al. also found that the recruitment of neutrophils to lymph nodes via this pathway requires CCR7: injection of complete Freund's adjuvant (CFA) failed to induce the rapid recruitment of neutrophils to the lymph nodes in CCR7-deficient mice[169].

1.3.3 Neutrophil Activation

Neutrophils are activated in response to a number of different stimuli, which promote prototypical neutrophil functions[170]. For instance, bacterial-derived formylated peptides, such as fMLF (N-formylmethionine-leucyl-phenylalanine), act via formyl peptide receptor 1 (FPR1) to trigger a range of intracellular kinase pathways that induce superoxide production, degranulation and chemotaxis[171]. Leukotrienes, which are generated by the metabolism of arachidonic acid through the 5-lipoxygenase pathway, act primarily on myeloid leukocytes to induce activation of integrins, adhesion to endothelial cell walls and chemotaxis[172]. Oyoshi and colleagues demonstrated that leukotriene B4-driven neutrophil recruitment to the skin is essential for allergic skin inflammation[172]. Platelet-activating factor (PAF) is another pro-inflammatory agent that activates neutrophils; PAF receptor antagonists were shown to prevent neutrophil migration across cytokine pre-treated endothelial cells[173]. Moreover, PAF primes neutrophils to respond to a secondary stimulus with increased ROS production[174]. The complement fragment C5a also induces actin-cytoskeleton polymerization and reorganization, thereby adjusting the mechanical properties of neutrophils and transforming them into migratory cells capable of being recruited to sites of inflammation[175]. Indeed, Sadik et al. determined that C5a plays a critical role in the initiation of neutrophil-mediated autoimmune inflammation in the arthritic joint[176].

1.3.4 The multifaceted functions of neutrophils

Neutrophils provide one of the first lines of defence against invading microorganisms[155]. They are highly mobile cells and quickly respond to inflammatory cues from infectious or damaged areas by trapping and killing invading pathogens[177]. Neutrophils are therefore traditionally known for their antimicrobial and phagocytic capabilities[177]. However, they possess a much more diverse repertoire of functional responses and display immunomodulatory properties as well[178].

1.3.4.1 Pathogen killing

Phagocytosis and the Respiratory Burst: Neutrophils rely upon several mechanisms for the eradication of invading microorganisms[177]. Phagocytosis refers to the uptake of a pathogen into a vacuole upon ligation of opsonic receptors[179]. This is followed by fusion with intracellular granules, the release of hydrolytic enzymes and the assembly of the NADPH oxidase complex within the membrane[179]. Activation of NADPH oxidase results in a dramatic increase in oxygen consumption (respiratory burst) and the production of superoxide anions (O_2-), which are further metabolized into hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS)[179]. These free radicals can induce damage to bacterial DNA and directly attack polyunsaturated fatty acids in membranes, resulting in decreased membrane fluidity and permeabilization[180]. However, neutrophils must precisely control the location and timing of NADPH oxidase activity because excessive ROS production can cause collateral tissue damage[163].

Degranulation: Neutrophil granules can also fuse with the plasma membrane, leading to the exocytosis of antimicrobial and cytotoxic molecules into the extracellular environment[181]. Degranulation is a highly regulated process that occurs in the reverse but ordered sequence of synthesis (formed-first-released-last model)[159,181]. Secretory granules require minimal cellular stimulation for release, whereas azurophilic granules require a very powerful agonist (e.g. fMLF) to promote degranulation and largely confine their release of proteolytic enzymes to the phagosome[159,182].

Degranulation of cationic host defence peptides, such as defensins and cathelicidins, plays an important role in antimicrobial responses[183]. These positively charged molecules interact with negatively charged cellular membranes, creating pores that can lead to permeabilization and the disruption of DNA/RNA biosynthesis[183]. However, neutrophil granule contents are also highly cytotoxic and can be detrimental to the surrounding tissue if secreted in an uncontrolled fashion[163]. For example, IL-6 has been shown to increase PMN cytotoxic potential through the selective enhancement of neutrophil elastase release in critically ill patients at risk for inflammatory organ injury[184].

NETosis: Another mechanism that neutrophils employ for pathogen eradication is NETosis, which can be induced by a variety of inflammatory stimuli (TNFα, IL-8, immune complexes) and PAMPs[185]. NETosis refers to the formation of neutrophil extracellular traps (NETs) and was first described by Brinkmann and colleagues in 2004[186]. NETs are important for the immobilization, neutralization and killing of invading microorganisms, thereby preventing their dissemination[187]. They consist of large, extracellular meshworks of decondensed chromatin fibres, decorated with cytosolic and granule-derived proteins such as MPO, neutrophil elastase, serine proteases, defensins and cathelicidins[186,188]. It has been suggested that NETs increase the effectiveness of these antimicrobial components by concentrating them in a fibrous network[186]. The lack of "eat me" signals on their cell surface distinguishes them from apoptotic neutrophils[185].

1.3.4.2 Immunomodulatory function of neutrophils

In recent years, it has become clear that neutrophils participate in complex bidirectional cross-talk with other immune cells[189]. Neutrophils receive signals from other leukocytes that modulate their own survival and function[190]. For example, T cell-derived cytokines such as IFNγ and GM-CSF greatly enhance neutrophil responsiveness to a secondary stimulus during activation[191]. In addition, both αCD3-activated $CD4^+$ and $CD8^+$ T cells were shown to increase neutrophil activation and delay their spontaneous apoptosis rate[192].

In turn, neutrophils can both amplify and suppress other effector responses via direct and indirect mechanisms[190]. For instance, they have been shown to promote DC maturation into

more effective antigen-presenting cells through direct contact-dependent mechanisms[193]. Moreover, neutrophil-macrophage interactions are important for both initiation and resolution of inflammation[189]. More specifically, neutrophils secrete several chemokines (CCL2, CCL3) and AMPs (cathelicidin) that recruit macrophages to sites of inflammation[194]. Neutrophils are also major producers of cytokines (BAFF, APRIL, IL-21) required for B cell survival, maturation and differentiation[189]. Conversely, Kamenyeva and colleagues demonstrated that neutrophil recruitment to the lymph nodes limits local humoral responses to *S. aureus*: neutrophil depletion increased the production of IgG and IgM in the lymph node following infection[195].

In addition, apoptotic neutrophils have been shown to possess anti-inflammatory potential and therefore play a role in the resolution phase of inflammation and in promoting tissue repair[196]. Neutrophils undergoing programmed cell death change their phenotype to produce 'eat me' signals that are recognised by surrounding phagocytes[196]. For example, annexin-1 is released by apoptotic neutrophils, which serves as a signal to initiate phagocytosis by macrophages[197]. Phosphatidylserine (PS) residues exposed on the surface of apoptotic cells interact with receptors on macrophages, which modifies the latter's transcriptional profile to increase production of immunoregulatory cytokines such as IL-10 and TGFβ[198].

If clearance does not follow apoptosis, secondary necrosis can occur in which cellular membrane integrity is lost, resulting in the release of harmful intracellular neutrophil mediators and subsequent exacerbation of inflammation[196]. However, necrotic neutrophils also possess anti-inflammatory potential[196]. For instance, Li and colleagues demonstrated that the human cathelicidin peptide, LL-37, induces secondary necrosis of neutrophils without promoting macrophage inflammation[199]. However, the authors of this study suggest that necrotic neutrophils may mediate host damage through the release of toxic granule contents under chronic or dysregulated conditions instead[199].

1.4 Neutrophil – T Cell Interactions

Cross talk between the innate and adaptive arms of the immune system is critical for the clearance of pathogens and the development of an effective memory response[200]. Historically, the differentiation and activation of T cells was thought to occur later than, and separate to, the innate response. However, neutrophils and T cells have been shown to co-exist in several acute and chronic inflammatory diseases. For example, one of the prominent features of EAE is the presence of a large number of neutrophils and early-infiltrating $CD3^+$ T lymphocytes in the spinal cord during the pre-clinical phase of disease[201]. Furthermore, both neutrophils and T cells were shown to co-localize for a short time during influenza and RSV infection (respiratory syncytial virus)[202,203]. Neutrophils and T cells can therefore be present at the same site and at the same time, although the implications of this are unknown.

1.4.1 Stimulatory effects of neutrophils on T cell behaviour

1.4.1.1 Neutrophils can act as antigen presenting cells

Neutrophils are capable of transporting antigen to sites of T cell priming[204]. For instance, Maletto and colleagues found that when FITC-labelled OVA was injected into the footpad of OVA/CFA (complete Freund's adjuvant) immunized mice, the main OVA^-FITC^+ cells recruited to the draining popliteal lymph nodes were neutrophils[205]. Similarly, neutrophils have been shown to rapidly migrate and shuttle live bacilli to the draining lymph nodes following *Mycobacterium bovis* BCG intradermal vaccination[206]. The migration of both human and mouse neutrophils to the lymphoid organs has been linked to the upregulation of CCR7; Beavillain et al. demonstrated that this migration was impaired in $CCR7^{-/-}$ knockout mice[207].

Besides simply transporting antigen to lymphoid organs, neutrophils are also capable of interacting with T lymphocytes and acting as professional APCs[208]. In a mouse model of acute graft-versus-host disease, neutrophils migrated to the mesenteric lymph nodes, where they co-localized with T cells to present alloantigen[209]. Abdallah and colleagues demonstrated that mouse neutrophils express MHC II and upregulate the co-stimulatory molecules CD80 and CD86 when co-incubated *in vitro* with $CD4^+$ T lymphocytes, resulting in increased T cell proliferation, IFNγ and IL-17A production[210]. Similarly, neutrophils sorted from the draining

lymph nodes of vaccinated rhesus macaques were able to present vaccine antigen to autologous antigen-specific memory $CD4^+$ T cells *ex vivo*[211]. In humans, neutrophils exposed to GM-CSF or IFNγ were also shown to upregulate MHC II expression in order to present *S. aureus* superantigens to $CD4^+$ T cells[212]. More recently, Polak et al. found that neutrophils isolated from the peripheral blood of birch pollen-allergic donors induced proliferative and cytokine responses of Bet v 1-specific effector T cells[213].

Neutrophils are also capable of cross-presenting antigens to $CD8^+$ T cells[208]. For example, one study used an *in vivo* model in which professional APCs do not express functional MHC I, to show that the injection of antigen-pulsed neutrophils induced the differentiation of naïve $CD8^+$ T cells into mature cytotoxic T cells[214]. Furthermore, Hufford and colleagues demonstrated that influenza-infected neutrophils can act as professional APCs for anti-viral $CD8^+$ T cells[215].

1.4.1.2 Neutrophils promote T cell responses by modulating dendritic cells

Neutrophils promote T cell responses by enhancing dendritic cell (DC) activation and migration through contact- and cytokine-dependent mechanisms[216]. For instance, neutrophils produce a number of chemokines that attract DCs[216]. *Toxoplasma gondii* infection triggers neutrophil synthesis of CCL3, CCL4, CCL5 and CCL20, all of which are strongly chemotactic for immature DCs[217]. Moreover, the early release of CCL3 by neutrophils in response to *Leishmania major* is involved in the first wave of DC recruitment to the site of infection[218]. Mycobacteria have also been reported to induce the release of DC chemoattractants: neutrophil depletion during *M. tuberculosis* infection delayed DC migration to the draining lymph nodes and as a result, impaired the protective anti-mycobacterial response[219]. Similarly, Weber and colleagues demonstrated that gelatinase is critical for the generation of contact hypersensitivity by promoting DC migration to the site of sensitization and subsequent allergen-specific T cell priming[220]. Cathepsin G and neutrophil elastase have also been shown to catalyse the removal of the C-terminal peptide from the inactive prochemerin, thereby releasing the active form, which is a potent chemoattractant for APCs[221].

Neutrophils strongly cluster with immature DCs and provide local co-factors that are required for their maturation into professional APCs[193]. More specifically, TNFα produced by activated neutrophils is essential for inducing DC maturation[193]. For instance, *T gondii*-stimulated neutrophils that release TNFα are responsible for the upregulation of CD40 and CD86 by DCs[217]. Similarly, the *in vitro* co-incubation of neutrophils with DCs exposed to *Aspergillus fumigatus* induces the upregulation of DC co-stimulatory molecules in a contact- and DC-SIGN-dependent manner[222]. Steinbach and colleagues demonstrated that CNS-infiltrating neutrophils secrete pro-inflammatory molecules and induce the maturation of bone-marrow-derived DCs *in vitro*, which in turn enhances their ability to re-stimulate myelin-specific T cells[223]. This implies that neutrophil-DC crosstalk plays a crucial role in the amplification of early CNS inflammation and the development of autoimmune disease[223].

DCs are also capable of acquiring antigen directly from phagocytosed neutrophils[216]. For example, they were shown to take up *C. albicans*-derived antigens from both live and apoptotic neutrophils and were thus capable of eliciting an antigen-specific T lymphocyte response[224]. Moreover, Blomgran et al. showed that DCs that acquired *M. tuberculosis* through the ingestion of infected neutrophils, migrated better to the draining lymph nodes compared to those that acquired the bacteria directly[219]. The authors suggested that this was a mechanism for the delivery of antigen to DCs, in a form that makes them more effective initiators of naïve $CD4^+$ T cell activation[219].

1.4.1.3 Neutrophils promote T cell activation and differentiation

Neutrophils can stimulate the immune response by directly priming T cells[204]. For example, NETs released by human neutrophils have been shown to reduce the activation threshold of T lymphocytes, resulting in increased proliferation and cytokine production in response to antigen[225]. Furthermore, Davey and colleagues demonstrated that ingestion of bacterial pathogens by human neutrophils and the subsequent release of microbial metabolites (HMB-PP) activates antimicrobial γδ T cells during early infection[226]. Several intracellular neutrophil mediators can also influence the cytokine microenvironment, which dictates how T cells respond[8]. For instance, proteinase-3 can process pro-TNFα to a biologically active soluble form, as well as directly activate pro-IL-1β[227]. Both TNFα and IL-1β enhance the expansion,

effector function, tissue localization and memory response of antigen-specific $CD4^+$ and $CD8^+$ T cells, thereby increasing and perpetuating the immune response[228].

In addition, neutrophils are capable of skewing T helper cell polarization[229]. For instance, rhinovirus infection in a mouse model of allergic airway hypersensitivity triggers double-stranded DNA release associated with NETs, which in turn promotes the development of type 2 immunopathology[230]. Conversely, McFarlane et al. found that neutrophils play a significant role in the development of a protective Th1 immune response during *Legionella pneumophilia* infection[231]. Neonatal neutrophils stimulated by group B Streptococcus have also been shown to induce a pro-inflammatory T helper cell bias, by promoting Th1 and Th17 characteristics in Tregs[232]. More recently, a study published by Krishnamoorthy et al. demonstrated that neutrophil cytoplasts present in the mediastinal lymph nodes activated lung DCs to specifically promote Th17 differentiation[233]. Another mechanism through which neutrophils promote Th17 responses is through the release of neutrophil elastase, which has been shown to process DC-derived CXCL8 into a truncated, potent Th17-inducing form[234].
Neutrophils can also influence the differentiation and effector functions of $CD8^+$ T lymphocytes. Neutrophil depletion during influenza infection results in impaired cytokine production and cytotoxic effector function of virus-specific $CD8^+$ T cells[235]. However, the absence of neutrophils has no effect on antigen presentation or the expansion of naïve $CD8^+$ lymphocytes[235].

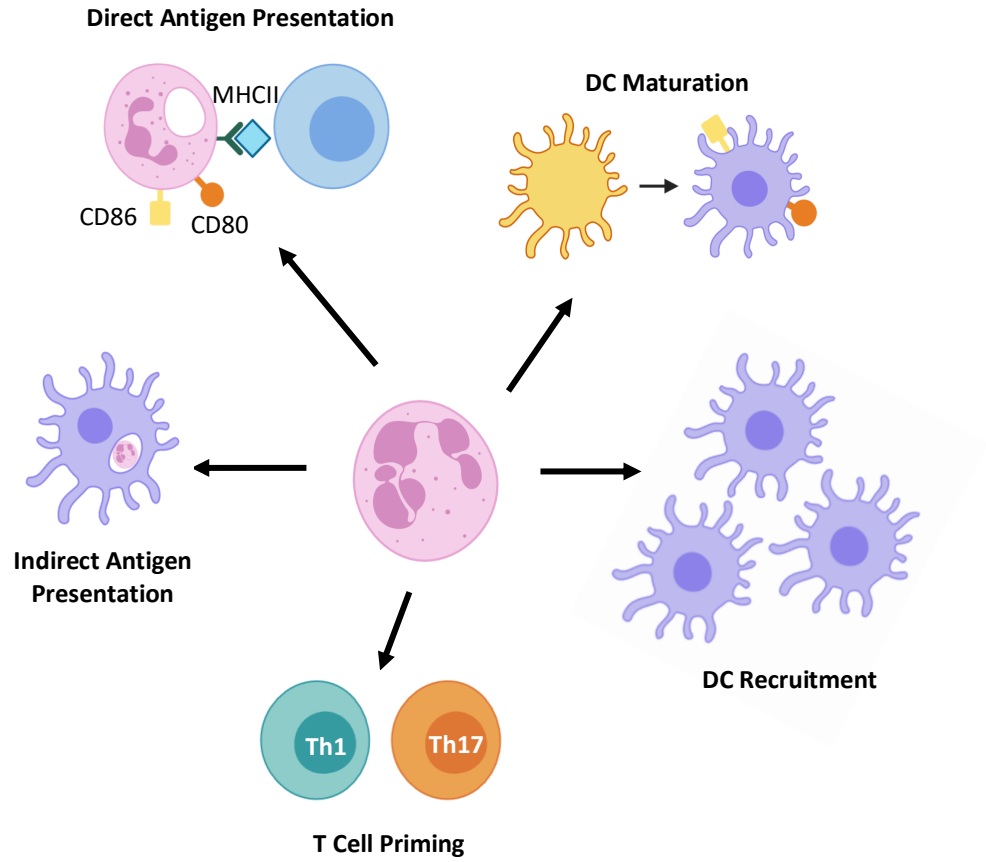


Figure 1.6: Mechanisms of neutrophil-mediated T cell stimulation. Neutrophils can directly present antigen to T cells by upregulating MHCII and co-stimulatory molecules such as CD80 and CD86. Dendritic cells can acquire antigen from phagocytosed neutrophils. Neutrophils skew T cell polarization towards a Th1/Th17 phenotype. Neutrophils can also promote T cell activation and differentiation by inducing DC recruitment and maturation.

1.4.2 Inhibitory effects of neutrophils on T cell behaviour

Neutrophils possess immunosuppressive mechanisms that inhibit T cell activation and proliferation (**Figure 1.7**)[204]. For instance, Tak and colleagues showed that immune suppression by neutrophils reduces T cell-mediated pathology after influenza infection[236]. Moreover, neutrophil depletion during *Brucella abortus* infection resulted in the more efficient elimination of the microbe due to enhanced B cell and Th1 responses[237]. Similarly, neutropenic mice infected with *Cryptococcus neoformans* displayed increased proportions of IL-17-producing γδ T cells in the lungs, suggesting that neutrophils exert a regulatory effect on this T cell subset as well[238].

1.4.2.1 Neutrophils produce immunosuppressive cytokines

Neutrophils can produce immunoregulatory cytokines that suppress the T cell response[204]. For instance, they have been shown to be significant producers of IL-10 during sepsis in mice[239]. The effects of IL-10 include the downregulation of key signalling receptors on APCs (CD40, CD80, CD86 and MHC II), suppression of T cell proliferation, IL-2, IL-6 and IFNγ production, and the maintenance of FOXP3 expression by Tregs[240]. Indeed, Doz et al. found that during mycobacterial infection, IL-10-producing neutrophils inhibit $CD4^+$ $IL\text{-}17^+$ lymphocytes, which they suggest is an important mechanism to control an otherwise exuberant and damaging Th17 response[241]. Moreover, both mouse and human neutrophils can produce TGFβ, which plays an essential role in establishing immune tolerance[195,242].

1.4.2.2 Neutrophil serine proteases inhibit T cell responses

Neutrophil serine proteases are traditionally known for their roles in the intracellular killing of pathogens[243]. However, they are also key regulators of the inflammatory response and can impair host defence by degrading immune receptors[229]. More specifically, Bank et al. demonstrated that neutrophil elastase was responsible for the selective proteolytic cleavage of CD25 and IL-6R at foci of inflammation, both of which play important roles in T cell activation[244].

In addition, neutrophil proteases can fine-tune the local inflammatory response through the disruption of gradients, or by modulating the biological activity of chemokines, cytokines and growth factors[245]. For example, CXCL12 is an important lymphocyte chemoattractant and its proteolytic cleavage by neutrophil elastase inactivates the chemokine and leads to decreased secondary recruitment of lymphocytes to the site of inflammation[246]. Furthermore, whilst proteinase-3 can process pro-TNFα to its biologically active soluble form, neutrophil elastase and cathepsin G have both been shown to degrade mature TNFα, resulting in a loss of activity[247]. Neutrophil-derived serine proteases have also been implicated in the degradation of pro-inflammatory IL-6, the inactivation of which could in turn dampen T cell responses[248].

1.4.2.3 Neutrophil-derived reactive oxygen species suppress T cells

Another mechanism through which neutrophils suppress T cells is the release of ROS, such as hydrogen peroxide (H_2O_2)[204]. H_2O_2 is the product of NADPH oxidase and potently inhibits T cell activation and proliferation[204]. For instance, Pillay et al. showed that in a model of acute inflammation induced by systemic challenge with LPS, a population of human neutrophils ($CD62L^{dim}CD11c^{bright}CD16^{bright}$) potently inhibits T cell proliferation through the highly localized release of H_2O_2 into the immunological synapse[249]. Furthermore, activated neutrophils inhibit DNA synthesis in human T lymphocytes proportionally to superoxide levels in the medium, which is associated with alterations in TCR signalling (downregulated TCRζ and decreased Nf-κB activation)[250].

Gelderman and colleagues found that mice that possess allelic polymorphisms in the *Ncf1* gene (neutrophil cytosolic factor 1, a component of neutrophil NADPH oxidase) have a lower capacity to produce ROS and are more susceptible to developing severe arthritis[251]. They suggested that ROS production is important in regulating surface redox levels of T cells and suppressing autoreactivity[251]. Severe systemic T cell suppression in patients with advanced cancer has also been attributed to granulocyte-derived H_2O_2[252]. For example, Mensurado et al. demonstrated that tumour-associated neutrophils suppress the proliferation and IL-17 production of $\gamma\delta$ T cells through the induction of oxidative stress[253]. Interestingly, the susceptibility of human T cells to H_2O_2-induced inhibition strongly depends on the T cell

subset: effector T cells are largely protected from ROS-mediated effects whereas memory T cells are the most vulnerable[254].

1.4.2.4 Arginase-1 impairs T cell activation and proliferation

Neutrophil tertiary (gelatinase) granules contain arginase-1, an enzyme that is inactive at physiological pH but activated by other factors released during primary granule release[159]. Arginase-1 catalyses the conversion of L-arginine to urea and L-ornithine, thus depleting the microenvironment of the former[255]. L-arginine is required for the expression of the invariant ζ chain of the TCR complex and its subsequent downregulation following depletion impairs T cell activation, function and proliferation[255]. Sippel et al. demonstrated that peripheral immunosuppression in post-stroke mice is mediated by neutrophil-derived arginase-1[256]. They observed decreased expression of CD3ζ on splenic T cells, which correlated with decreased functional activity[256]. Mature neutrophils from the cystic fibrosis airway have also been shown to suppress T cell activation via arginase-1[257].

Another mechanism through which arginase-1 reduces T cell function is cell cycle arrest: activated T cells cultured in the absence of L-arginine are arrested in the G0-G1 phase[258]. Rodriguez et al. proposed that L-arginine starvation results in the inhibition of protein synthesis and the inability of T cells to upregulate cyclin D3[258]. In addition, arginase-1 impairs the formation and stability of the immunological synapse between T cells and DCs, thereby suppressing T cell activation[259]. This is mediated by the inhibition of cofilin dephosphorylation, which leads to altered actin polymerization[259].

Neutrophils play an important role in regulating L-arginine levels during normal human pregnancy, as they express increased levels of arginase-1 in the blood and placenta[260]. This is associated with T cell hyporesponsiveness and may contribute to tolerance of the semi-allogenic fetus[260]. Moreover, neonatal neutrophils have a higher arginase-1 content than adult neutrophils and are therefore more suppressive[261]. This could provide an explanation as to why newborns are more susceptible to infection[229].

1.4.2.5 The PD1/PDL1 pathway

PD1 is one of several co-inhibitory T cell receptors that regulate T cell responses[262]. Binding of PD1 to its ligand, PDL1, delivers a negative signal that contributes to the cessation of T cell proliferation[262]. Neutrophils have been shown to upregulate PDL1 under various conditions[178]. For example, Wang et al. found that neutrophils increase PDL1 expression during sepsis, which they postulated may be related to sepsis-induced immunosuppression[263]. Furthermore, neutrophils isolated from the peripheral blood of patients infected with *Burkholderia pseudomallei* also displayed increased expression of PDL1, which was responsible for inhibiting polyclonal T cell activation[264]. Similarly, HIV-1 virions are potent inducers of neutrophil PDL1 expression[265]. Increased neutrophil PDL1 is increased in individuals with HIV and correlates to the dampening of T cell responses[265].

The PD1/PDL1 axis has been heavily implicated in cancer[262]. Neutrophils that accumulate in gastric cancer tissues express higher levels of PDL1 and effectively inhibit the proliferation and activity of $PD1^+$ T cells *in vitro*[266]. This dampens T cell-mediated anti-tumour immune responses and has therefore been associated with poor patient survival[266]. In addition, Cheng et al. discovered that cancer-associated fibroblasts induce $PDL1^+$ neutrophils that foster immune suppression in hepatocellular carcinoma[267].

1.4.2.6 Neutrophils suppress dendritic cell maturation and function

Whilst neutrophils can positively regulate DCs, they are also capable of suppressing their maturation and function[216]. For instance, supernatants from sputum isolated from patients with chronic obstructive pulmonary disease (COPD) and cystic fibrosis suppress the expression of DC costimulatory molecules (including CD40, CD80 and CD86), in a neutrophil elastase-dependent manner[268]. Moreover, Odobasic et al. demonstrated that neutrophil MPO regulates T cell−driven tissue inflammation in mice by inhibiting DC function[269]. More specifically, MPO inhibits DC activation and migration to the lymph nodes by reducing their expression of CCR7[269].

Neutrophils have also been shown to compete with DCs at the level of antigen presentation[204]. Yang and colleagues found that neutrophils modulate antigen presentation

following immunization in various adjuvants and that the $CD4^+$ T and B cell responses are enhanced in neutropenic mice[270]. In addition, MPO was found to inhibit antigen uptake and processing by DCs in a model of antigen-induced arthritis[269].

1.4.2.7 Neutrophils promote Treg development and function

Neutrophils modulate the behaviour of regulatory T cells by promoting their development, recruitment and immunosuppressive activity[229]. For example, neutrophil elastase acts specifically on immature human DCs to turn off IL-6 production and increase TGFβ, a cytokine that is critical for Treg development[271]. Indeed, Tateosian et al. found that neutrophil elastase-treated DCs promote the generation of suppressive $CD4^+$ $FOXP3^+$ T cells *in vitro*[271]. Similarly, lactoferrin acts directly on T lymphocytes to upregulate Treg-specific genes and skew differentiation towards a regulatory phenotype[272]. *In vivo*, this rescued normal intestinal physiology by enhancing gut barrier function in several models of IBD[272]. Finally, Mishalian et al. demonstrated that neutrophil-derived CCL17 recruits regulatory T cells into tumours, thereby impairing anti-cancer immunity[273].

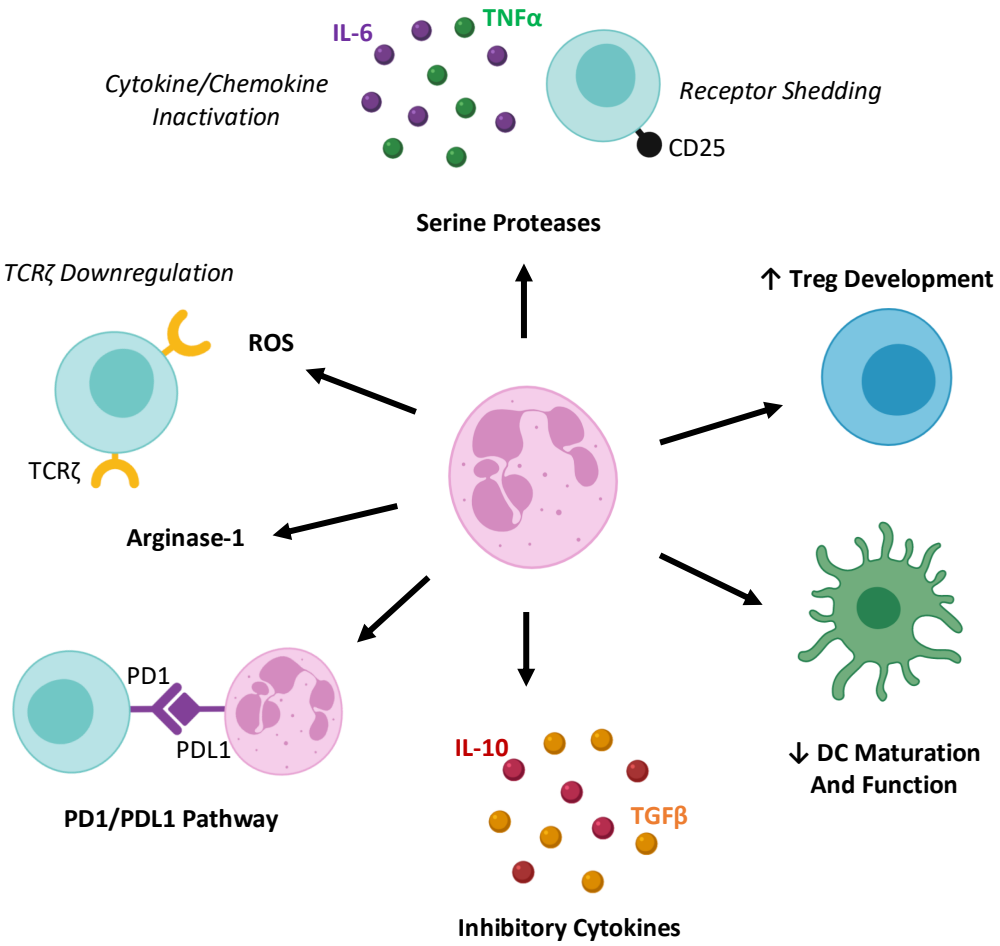


Figure 1.7: Mechanisms of neutrophil-mediated T cell suppression. Neutrophils secrete inhibitory cytokines and serine proteases that inactivate pro-inflammatory cytokines, disrupt chemokine gradients and promote the shedding of receptors such as CD25. The production of ROS and arginase-1 inhibit T cell activation and proliferation by downregulating TCRζ signaling. Neutrophils upregulate the expression of PDL1 and deliver suppressive signals upon binding to its receptor on T cells. Neutrophils suppress DC activation and compete with them for antigen presentation. Neutrophils also promote the development and immunosuppressive activity of regulatory T cells.

1.4.3 Conflicting data of how neutrophils affect T cell behaviour

Extensive research investigating how neutrophils affect T cell behaviour has yielded conflicting and confusing data: neutrophils can both suppress and promote T cell activation, depending on the context[204,229]. It remains to be seen whether these differential effects are mediated by discrete neutrophil populations that have not yet been stratified, or by pre-existing neutrophils that acquire immunomodulatory properties under specific pathophysiological conditions[208]. For example, it has been suggested that the maturation status of neutrophils dictates their inhibitory or stimulatory phenotype: those released from the bone marrow during sepsis are immature and suppressive to T cells, whereas neutrophils present during hyperlipidemia are primed and produce high levels of MPO[249].

Other possibilities that can explain these wildly different outcomes include 1) the inflammatory model or infection used 2) differences between human and mouse neutrophils and 3) experimental techniques employed[229]. For instance, low-density neutrophils have been shown to find their way to the PBMC (peripheral blood mononuclear cells) layer following density gradient centrifugation, a popular method used for the isolation of neutrophils[274]. Low-density neutrophils may represent granulocytic myeloid-derived suppressor cells (G-MDSC), which impair T cell proliferation *in vitro*[274]. It is virtually impossible to distinguish MDSC from mature neutrophils and contamination of PBMCs with this subset can skew experimental readouts[155,274]. Furthermore, neutrophils have been shown to phagocytose αCD3/CD28-coated activation beads, which are commonly used to activate T cells *in vitro*[274]. This can result in the artefactual suppression of T cell proliferation[274].

1.5 Cathelicidin

Antimicrobial host defence peptides (AMP or HDP) are a large group of relatively small, cationic molecules that constitute a highly conserved component of the innate immune system and are traditionally known for their ability to kill microbes, including bacteria, viruses, fungi and protozoa[183]. Mammalian AMPs can be subdivided in to two major families: the defensins and cathelicidins[183].

1.5.1 The discovery of cathelicidin

The first cathelicidin, cecropin, was isolated in 1980 from the *Hyalophora cecropia* moth[275]. Magainin was isolated in 1987 by Zasloff and colleagues from the skin of the *Xenopus leavis* frog[276]. The first mammalian cathelicidins discovered were rabbit CAP18 and the bactenecins, which were isolated from bovine neutrophils[277,278]. However, since then, around 30 cathelicidin family members have been identified in multiple mammalian species, including humans, monkeys, mice, rats, pigs and cattle[277]. Humans and mice possess only a single cathelicidin, whereas pigs and cattle possess multiple genes encoding this HDP[277]. Human cathelicidin, human cationic antimicrobial peptide 18 (hCAP18), was first isolated from neutrophils in 1995[279]. Mouse cathelicidin, or mCRAMP (cathelin-related antimicrobial peptide) was identified in 1997 by Gallo et al.[280]. The cathelicidin family is highly heterogenous: peptides can display a variety of different structures and amino acid sequences[281]. However, they all possess a conserved cathelin domain, which shows very high interspecies homology[281].

1.5.2 Cathelicidin expression and regulation

1.5.2.1 Cathelicidin expression

Cathelicidins can be found in a wide variety of tissues, typically at mucosal surfaces that are in direct contact with the environment[281]. These include the airways, gastrointestinal mucosa and reproductive tract, where they play a role in early host defence[281]. Cathelicidins are produced by a variety of cells, including neutrophils, epithelial cells, keratinocytes, macrophages, NK cells and mast cells[282]. Depending on the cell type, cathelicidin expression

is either constitutive, or induced in response to infection, injury and/or inflammation[277]. For instance, cathelicidin is up-regulated in skin in response to cutaneous injury or infection with group A *Streptococcus*[265]. Furthermore, whilst *CAMP* is not expressed in normal, healthy skin, it is induced in human keratinocytes during inflammatory disorders such as lesional psoriasis[266].

Cathelicidin can also be detected in numerous secretions, including sweat, wound and airway surface fluids, as well as seminal plasma, where it provides a sterile environment during fertilization[281]. For example, the average concentration of human cathelicidin in broncho-alveolar lavage (BAL) fluid is around 5 μg/mL in healthy individuals[285]. However, this can rise to 30 μg/mL in cystic fibrosis patients[285].

The human and mouse cathelicidin genes (*CAMP*) have been mapped to chromosomes 3 and 9, respectively[277]. Cathelicidins are encoded by genes consisting of four exons and are produced as inactive pre-pro-peptides[277]. In neutrophils, cathelicidin is produced at the myelocyte and metamyelocyte stages of maturation[277,286]. The first exon encodes the "pre-" signal peptide, whilst exons two and three encode the highly conserved "pro-" N-terminal cathelin domain[277,285]. The cathelin domain is made up of approximately 100 residues and shows great interspecies homology[277,285]. Exon 4 encodes the mature C-terminal peptide, consisting of the antimicrobial sequence (**Figure 1.8**)[277,285].

Inactive cathelicidin precursors must be proteolytically processed to release the biologically active C-terminal domain[277]. This was first illustrated by Zanetti and colleagues who found that bovine bactenecins are generated from precursor molecules and require proteolytic cleavage by neutrophil azurophilic serine proteases, later found to be neutrophil elastase[287,288]. In humans, the extracellular processing of neutrophil-derived hCAP18 is mediated by proteinase-3 and liberates a linear 37 amino acid-long peptide, LL-37[289]. The segregation of hCAP18 and proteinase-3 within different granules prevents unwanted intracellular processing of AMPs in resting neutrophils[281].

On the other hand, epididymal-derived hCAP18 in seminal plasma is cleaved by the prostate-derived gastricsin (pepsin C) when exposed to vaginal fluid at low pH[281,290]. Furthermore, Yamasaki et al. identified novel cathelicidin peptide forms at the skin surface that were

generated following proteolytic processing by the serine protease, kallikrein 5[291]. Abnormal production of this enzyme within the epidermis and the presence of these cathelicidin peptides was subsequently shown to promote skin inflammation in rosacea[292]. In addition, proteases in human sweat can cleave LL-37 to smaller peptides at the skin surface, which increases their antimicrobial activity but alters their immunostimulatory capacity to induce IL-8 release from keratinocytes[293]. This demonstrates the importance of proteolytic processing on the activity of cathelicidins[291].

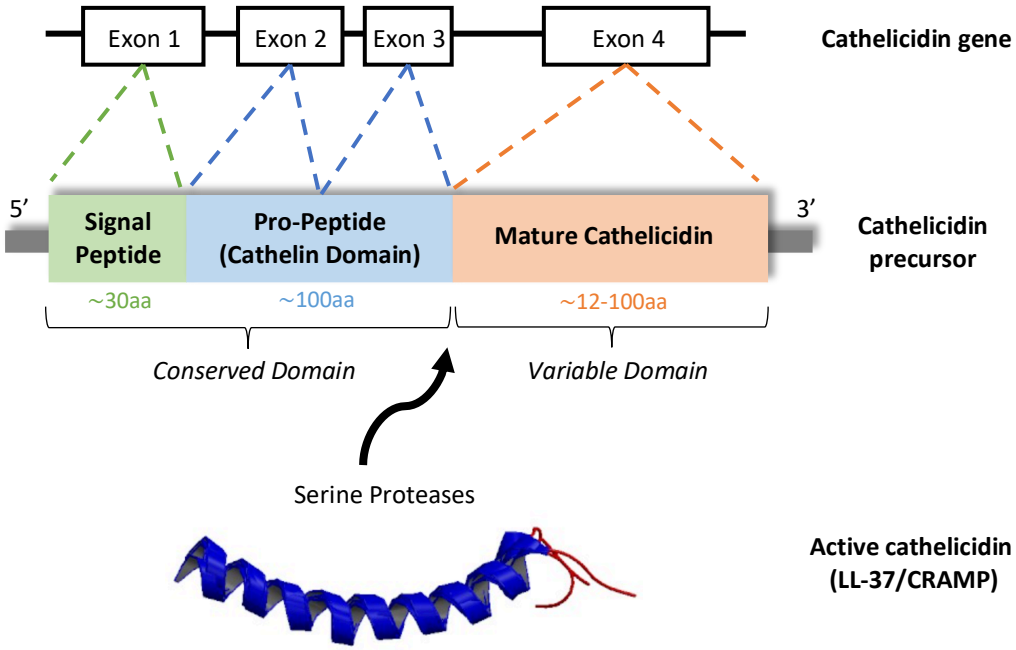


Figure 1.8: **Cathelicidin expression and maturation**. Cathelicidins are encoded by genes made up of 4 exons. Cathelicidins are produced as inactive precursors and must be proteolytically cleaved to release the biologically active peptide. The cathelin domain is highly conserved, whilst the C-terminus is highly variable. Both human (LL-37) and mouse (CRAMP) cathelicidin adopt an α-helical structure.

1.5.2.2 Regulation of cathelicidin expression

Vitamin D deficiency has been linked to an impaired immune response and increased susceptibility to infection[294]. One of the major regulators of human cathelicidin expression is vitamin D[294]. The 5'-upstream promoter region of *CAMP* possesses a Vitamin D Response

Element (VDRE) sequence that binds to active 1,25-dihydroxyvitamin D_3 and several of its analogues[295]. Gombart and colleagues demonstrated that the *in vitro* stimulation of human epithelial cells and monocytes with vitamin D_3 increased LL-37 expression[296]. However, induction of *CAMP* in mouse cells was not observed and they postulated that this was due to the evolutionary loss of their VDRE[296]. In addition, TLR2 activation following bacterial challenge increases the expression of the CYP27B1 hydroxylase enzyme in keratinocytes, which is responsible for the catalytic conversion of the inactive vitamin D pro-form and subsequent upregulation of cathelicidin expression[297]. However, there is little evidence that suggests vitamin D increases cathelicidin expression *in vivo*. Furthermore, many of the benefits that some have speculated are due to the effects of vitamin D on the expression of this peptide could in fact be related to its regulation of other processes, including skewing T cell differentiation away from a pro-inflammatory Th17 phenotype and facilitating the induction of regulatory T cells[298].

Several vitamin D-independent regulatory mechanisms have also been identified[282]. For instance, Park et al. found that the perturbation of the epidermal permeability barrier induces ER stress, which in turn stimulates the synthesis of cathelicidin in epithelial cells, thereby restoring or enhancing antimicrobial defence barriers[299]. Cathelicidin expression can also be regulated by various endogenous factors such as pro-inflammatory cytokines and growth factors[282]. The *CAMP* gene contains promoter elements that are potentially regulated by IL-6, and a co-localization between IL-6 and hCAP18 has been observed in squamous epithelial tissue, suggesting a role for this peptide in epithelial antimicrobial defense[300].

Other signals, such as glucocorticoids produced in response to psychological stress, suppress cathelicidin expression[301]. Interestingly, calcipotriol, a synthetic derivative of vitamin D, negatively regulates cathelicidin in cells cultured under inflammatory conditions[302]. Moreover, certain pathogens, such as *Shigella flexneri*, are also capable of downregulating cathelicidin in intestinal epithelial cells during early infection as part of a strategy to circumvent immune defences and increase virulence[303].

1.5.3 Cathelicidin structure

Biologically active cathelicidin peptides are highly heterogenous: they vary in amino acid sequence and size and display considerable structural diversity[277]. Like most AMPS, cathelicidins display an overall positive charge, ranging from +2 to +9, due to the presence of a high number of positively charged amino acids, such as lysine and arginine[183]. Furthermore, up to 50% of the structure is made up of hydrophobic resides[183]. Most cathelicidins are therefore linear peptides that adopt an amphipathic α-helical structure in environments mimicking biological membranes (e.g. LL-37, mCRAMP)[281,304]. Others possess a β-hairpin motif (e.g. protegrin-1), whilst some contain a high number of tryptophan residues (e.g. indolicidin), or form extended polyproline-type structures if proline/arginine rich (e.g. bactenecins)[277,281,304]. Small changes in the charge, helicity and hydrophobicity of cathelicidins can have a significant impact on their activity: a single amino acid change within the sequence can dramatically alter the anti-bacterial and/or toxicity profile of a peptide[305].

1.5.4 The antimicrobial effects of cathelicidin

The C-terminal peptide of cathelicidin exhibits broad antimicrobial activity against both Gram-positive and Gram-negative bacteria, enveloped viruses and fungi[304]. As such, cathelicidin knockout mice are prone to developing infections of the skin, lungs, gastrointestinal and urinary tract, as well as the eyes[306–309]. For example, mCRAMP-deficiency predisposes animals to skin infection by group A *Streptococcus* and low levels of hCAP18 have been detected in skin lesions from patients with atopic dermatitis, who display increased susceptibility to skin infections[306,310]. Furthermore, neutrophils from individuals with morbus Kostmann syndrome (or severe congenital neutropenia) exhibit LL-37 deficiency (and reduced concentrations of α defensins)[311]. No LL-37 could be detected in the plasma or saliva of these patients, which correlated with increased recurrent chronic periodontal disease[311].

1.5.4.1 Anti-bacterial

The mixed cationic and hydrophobic composition of cathelicidins enables them to interact with and permeabilize microbial cytoplasmic membranes, which typically present anionic surfaces, rich in lipids[312]. Gross disruption of the microbial membrane structure and

morphology is often noted following treatment with cathelicidin, including membrane blebbing and fragmentation[312]. Several studies have determined that cathelicidin creates trans-bilayer toroidal pores to disrupt bacterial cell wall integrity and induce cell death[313]. More specifically, monomers and oligomers cover the surface of the lipid bilayer, with positively charged amino acids binding to the head groups of negatively charged phospholipids[314]. The accumulation of peptides causes a positive curvature strain, which induces the transient formation of small toroidal pores[313]. This results in a loss of transmembrane potential, ultimately causing leakage of cytoplasmic components and rendering intracellular processes vulnerable[314]. For example, cathelicidin can impair RNA/DNA synthesis and enzyme activity, as well as promote protein degradation[285].

Cathelicidins exhibit several other anti-bacterial functions[285]. For example, Kirikae et al. demonstrated that human cathelicidin possesses LPS-neutralizing activity and was capable of preventing antibiotic-induced endotoxic shock in mice with septicemia[315]. Furthermore, cathelicidins are readily incorporated into NETs *in vivo*, where they stabilize the structure and contribute to their antimicrobial effects[285].

Finally, low concentrations of LL-37 prevent the formation of *Pseudomonas aeruginosa* biofilms[316]. Microarray studies revealed that cathelicidin downregulates the expression of quorum-sensing-controlled genes and upregulates type IV pili, thereby increasing twitching behaviour and impairing attachment of the bacteria to each other and/or other surfaces[316]. As such, immobilized or gel-trapped cathelicidins are commonly used to coat medical devices such as catheters to prevent the formation of biofilms[285].

1.5.4.2 Anti-viral

Cathelicidin exhibits potent anti-viral activity[285]. Both LL-37 and mCRAMP reduce disease severity and viral load in mice infected with influenza A virus (IAV)[317]. More specifically, animals treated with therapeutic cathelicidin had lower concentrations of pathogenic, pro-inflammatory cytokines in the lung compared to untreated controls[317]. However, this was not a result of direct anti-viral activity. Conversely, Currie and colleagues demonstrated that LL-37 prevented respiratory syncytial virus (RSV)-induced cell death in epithelial cultures by

significantly inhibiting the production of new infectious particles and reducing the spread of infection[318]. Similarly, Bergman et al. discovered that LL-37 is capable of inhibiting the replication of HIV *in vitro* in primary $CD4^+$ T cells and cell lines[319].

Electron microscope studies have shown that LL-37 exerts its effects, in part, by disrupting the viral membrane, thereby impairing viral survival and propagation within infected cells[320]. For instance, Dean et al. found that human cathelicidin removes the outer membrane of vaccinia virus (VV), which results in exposure of normally sequestered antigens and viral susceptibility to antibody neutralization[321].

1.5.4.3 Anti-fungal

Cathelicidin possesses anti-fungal activity against multiple fungi, including *C. albicans*[322]. Human LL-37 and mouse mCRAMP have both been shown to kill this fungus by permeabilizing the membrane and inhibiting fungal growth[322]. LL-37 induces rapid phase separation and disintegration of the *C. albicans* membrane into discrete vesicles, resulting in the formation of large pores and the instantaneous efflux of small molecules such as ATP[323]. However, it has been suggested that this is not the sole cause of death: increased membrane permeability may lead to the uptake of the peptide and intracellular effects[324]. For example, Ho Wong and colleagues found that reactive oxygen species could be detected in the yeast form of *C. albicans* after treatment with active human cathelicidin fragments[324].

In addition, Luo et al. recently showed that synthetic LL-37 directly binds to the surface of *A. fumigatus* and disrupts the integrity of the cell wall[325]. LL-37 inhibits mycelial growth in a concentration-dependent manner and significantly attenuates mycelial adhesion and the invasion/destruction of epithelial cells[325]. Moreover, cathelicidin-overexpressing mice are less susceptible to pulmonary *A. fumigatus* infection compared to wildtype animals[325].

1.5.5 The immunomodulatory effects of cathelicidin

As well as exerting powerful antimicrobial effects, cathelicidins play a significant immunomodulatory role[285]. They are capable of directly and indirectly recruiting, activating

and suppressing both innate and adaptive cells to shape the immune response (**Figure 1.9**)[282,285].

1.5.5.1 Cathelicidin acts through multiple receptors

Cathelicidins are both immunostimulatory and immunoregulatory[282,285]. One explanation for the plethora of effects exerted by cathelicidin is that they are driven by the activation of multiple receptors, dependent on cell type and context[326]. For example, LL-37 can activate Formyl Peptide Receptor 2 (FPR2), a pertussis toxin-sensitive G-protein coupled receptor (GPCR), to recruit peripheral blood neutrophils, monocytes and T cells to sites of inflammation[327]. However, several groups have shown that specific FPR2 blockers, such as WRW4, could not abrogate the effect of LL-37 under certain conditions[326]. Several other GPCRs have consequently been suggested to mediate the effects of cathelicidin, including the IL-8 receptor CXCR2: Zhang et al. demonstrated that binding of LL-37 to CXCR2 on neutrophils induced intracellular calcium mobilization and promoted their migration[328].

Alternative receptors of cathelicidin include the purinergic P2X7 receptor (P2X7R), which binds to extracellular ATP and whose activation leads to inflammasome formation and the processing of IL-1β and IL-8[329]. For example, the ability of LL-37 to stimulate fibroblast growth was inhibited by P2X7R inhibitors[330]. However, the precise mechanism through which LL-37 activates P2X7R remains unknown[326]. It has been suggested that unlike GPCRs who generally require a structurally unique ligand, the interaction with P2X7R could be a consequence of cathelicidin inserting itself into the membrane and interacting with the C' terminal end of the receptor[326]. In a similar vein, Tjabringa and colleagues demonstrated that LL-37 promotes innate immunity at the airway epithelial surface by transactivating the epidermal growth factor receptor (EGFR) via metalloproteinase-mediated cleavage of membrane-anchored EGFR-ligands[331]. In addition, transactivation of EGFR by LL-37 was also shown to contribute to wound healing by inducing keratinocyte migration[332].

In addition, cathelicidin can be taken up by cells in a non-specific manner[333,334]. For instance, LL-37 can bind to extracellular DNA plasmids through electrostatic interactions and target them to the nuclear compartment of mammalian cells (human embryonic fibroblasts, bladder carcinoma cells), in a process that is dependent on lipid rafts and proteoglycans[333].

Furthermore, Lande and colleagues demonstrated that LL-37 helps break innate tolerance and drives autoimmunity in psoriasis by binding to self-DNA to form aggregated and condensed structures that are delivered to and retained within early endocytic compartments in plasmacytoid DCs (pDC)[334]. This triggers TLR9 and interferon production[334]. In both studies, a specific receptor was not required.

1.5.5.2 The pro-inflammatory effects of cathelicidin

Cathelicidins promote the expression of pro-inflammatory cytokines and cytokine receptors[285]. For instance, LL-37 increases blood monocyte polarization of macrophages towards a more pro-inflammatory M1 phenotype[335]. van der Does et al. found that even the treatment of fully matured M2 macrophages with LL-37 for 6 days resulted in an enhanced M1 pro-inflammatory cytokine signature upon LPS stimulation[335].

Cathelicidins can also influence the maturation and function of DCs[285]. LL-37 stimulates the differentiation of monocytes into immature DCs, which express higher levels of HLA-DR and the co-stimulatory molecule, CD86[336]. Similarly, LL-37 promotes the very rapid and highly efficient sensing of CpG motifs in bacterial DNA by human pDCs[337]. This results in the upregulation of CD40/CD86 and increased IL-6 production, thereby promoting rapid responses to invading microbes[337]. Gwyer Findlay et al. demonstrated that DCs generated in the presence of human cathelicidin enhance the proliferation, activation and cytokine production of $CD8^+$ T cells, which in turn promote tumour regression in established squamous cell carcinomas in mice[338].

Cathelicidin can act as a T cell autoantigen[285]. Two-thirds of patients with moderate to severe plaque psoriasis harbour LL-37-specific T cells, which produce significant amounts of IFNγ and IL-17[339]. The presence of circulating LL-37-specific T cells correlates with disease activity, suggesting a contribution to disease pathogenesis[339]. It is thought that high levels of LL-37 bind to self-DNA released by dead cells in psoriatic lesions, forming a complex that activates intracellular TLRs within pDCs[334]. This breaks tolerance to self-antigens and promotes a strong IFNα response, resulting in the maturation of conventional DCs and the subsequent pathogenic proliferation of Th1 cells[334].

Cathelicidins can also influence T cell proliferation and differentiation[285]. Thomi and colleagues demonstrated that LL-37 increased the proliferation of healthy, human $CD4^+$ T cells *in vitro*[340]. Moreover, cathelicidin can act as an immune adjuvant: immunisation with OVA and mCRAMP led to increased splenocyte proliferation, as well as enhanced cytokine production and OVA-specific humoral responses[341]. In addition, LL-37-derived DCs display enhanced secretion of Th1 inducing cytokines such as IL-12, and are therefore capable of promoting Th1 differentiation *in vitro*[336]. LL-37 is localized inside the nucleus after it has been endocytosed and some have postulated that it can act directly as a transcription factor or enhancer to skew T cell polarization thanks to its ability to bind DNA and RNA[285,342].

It is important to note that the pro-inflammatory effects of cathelicidin can be beneficial to the host. Beaumont and colleagues demonstrated that the delivery of exogenous synthetic cathelicidin enhanced a protective pro-inflammatory response to *P. aeruginosa* lung infection, which promoted bacterial clearance from the lung in the absence of direct microbicidal activity[343]. More specifically, cathelicidin enhanced the development of an early pulmonary neutrophil response, which was absent in cathelicidin-deficient mice, resulting in significantly impaired pathogen clearance[343].

1.5.5.3 The anti-inflammatory effects of cathelicidin

Cathelicidin stimulates the production of anti-inflammatory cytokines by a variety of cell types[285]. For instance, several studies have shown that LL-37-stimulated monocytes and macrophages produce IL-10 and upregulate their expression of TGFβR[285]. Furthermore, Choi et al. demonstrated that human cathelicidin enhances PBMC production of IL-1RA, a well-known IL-1β antagonist, which inhibits IL-1β and TNFα production by IL-32 induced inflammatory monocytes[344]. Similarly, Luo and colleagues found that treatment of *A. fumigatus*-stimulated macrophages with LL-37 results in the downregulation of pro-inflammatory cytokines such as IL-6 and TNFα[325].

Thanks to its cationic and amphipathic properties, extracellular cathelicidin can directly bind and neutralize negatively charged, hydrophobic LPS molecules, preventing its interaction

with TLR4 and suppressing endotoxin-mediated upregulation of inflammatory cytokines[285,345]. For example, TNFα release by human monocytes stimulated with LPS *in vitro* is dramatically reduced upon treatment with low, physiological concentrations of LL-37 (≤1 μg/mL)[346]. In addition, the inhibition of TLR4 signalling in DCs by cathelicidin suppresses priming by LPS (downregulated HLA-DR, CD80, CD86) and the subsequent proliferation and differentiation of T lymphocytes (less IL-2 and IFNγ)[347]. mCRAMP was also found to constrain allergic contact dermatitis by inhibiting TLR4-induced cytokine release and upregulation of co-stimulatory molecules such as CD40, CD80 and CD86, presumably by binding and neutralizing LPS[348].

Taken together, one could speculate that high concentrations of cathelicidin at the site of infection *in vivo* serve to directly kill the invading pathogen, whilst simultaneously neutralizing endotoxins released as a result of this killing[285]. This could prevent excessive immune activation and reduce the chance of septic shock[285].

1.5.5.4 Chemotaxis

Cathelicidin can act as a direct chemoattractant to recruit both innate and adaptive immune cells to sites of inflammation[285]. For instance, LL-37 signals via FPR2 to attract a variety of leukocytes including neutrophils, eosinophils and monocytes[349,350]. However, it does not recruit monocyte-derived immature DCs through this mechanism because they downregulate FPR2 during differentiation[351]. Interestingly, while $CD4^+$ T cells migrate towards LL-37, $CD8^+$ T lymphocytes do not[352].

Cathelicidins can also indirectly promote chemotaxis by stimulating the expression of chemokines and chemokine receptors[285]. For example, several immune cells upregulate the expression of CCL2 in response to cathelicidin, including neutrophils, monocytes and macrophages, which in turn recruits T cells and DCs to the site of inflammation[285,353]. Furthermore, both LL-37 and mCRAMP induce CXCL10 expression, which attracts macrophages, T cells, NK cells and DCs[354]. Human cathelicidin also induces the production of IL-8 by macrophages, which stimulates neutrophil migration[285,353].

1.5.5.5 Apoptosis

Cathelicidin suppresses apoptosis of several cell types during inflammation, including epithelial cells and neutrophils[326]. For instance, Nagaoka et al. demonstrated that LL-37 prolongs the lifespan of neutrophils by suppressing apoptosis via the activation of FPR2 and P2X7R[355]. LL-37 has also been shown to provide anti-apoptotic signals to intestinal epithelial cells to maintain barrier integrity[356]. The inhibition of apoptosis results in a prolonged period where these cells are able to produce chemokines and cytokines, as well as clear microbes from the site of infection[326]. However, uncontrolled regulation of apoptosis can cause disease[326]. Systemic sclerosis patients display increased levels of LL-37, which correlate with their fibroblasts' inability to undergo apoptosis[357]. Similarly, Chamorro and colleagues suggested that the overexpression of LL-37 may contribute to reduced keratinocyte apoptosis in conditions such as psoriasis[358].

Conversely, several reports suggest that cathelicidin promotes apoptosis of certain cell types[359,360]. For example, Mader and colleagues demonstrated that LL-37 induces granzyme-mediated apoptosis of regulatory T cells and cytotoxic $CD8^+$ T lymphocytes[359,360]. However, it is important to note that the concentration of cathelicidin used in these studies was very high (40 μg/mL) and therefore potentially not physiologically relevant.

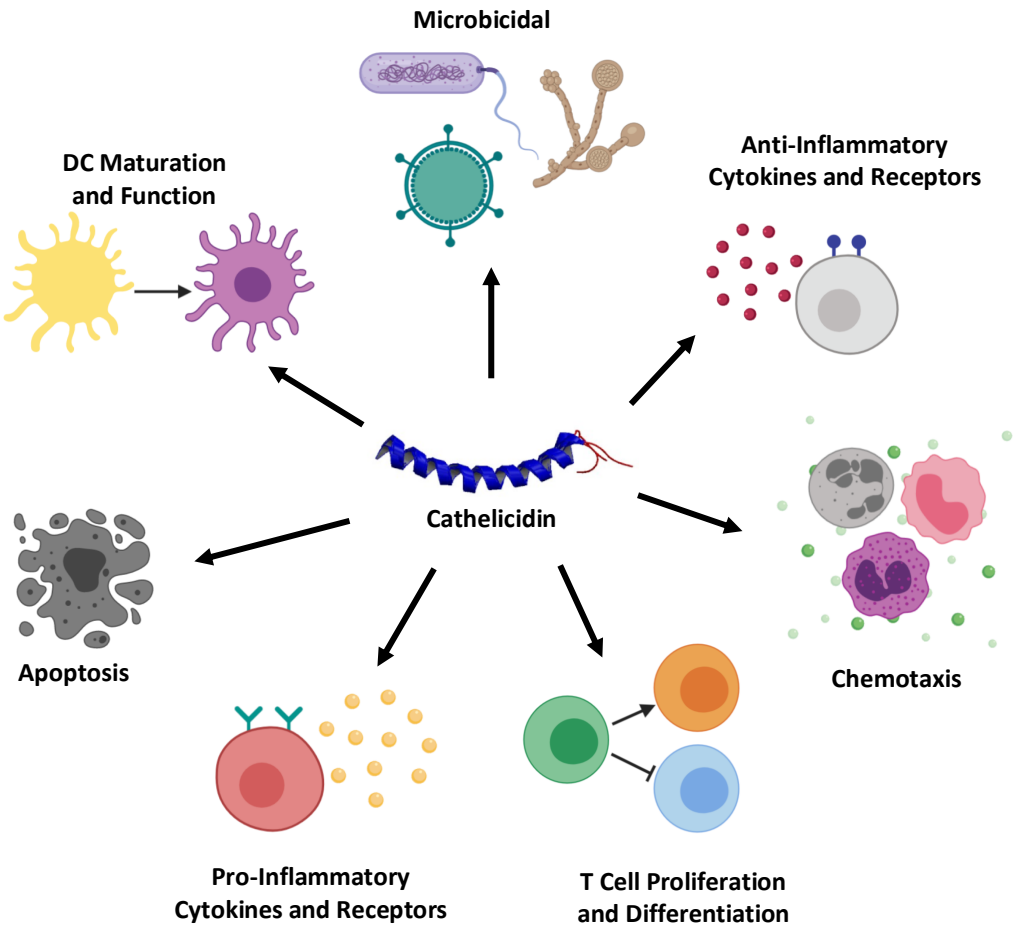


Figure 1.9: **The biological effects of cathelicidins**. Cathelicidins directly kill bacteria, viruses and fungi. Cathelicidins induce the production of both anti-inflammatory and pro-inflammatory cytokines and receptors. Cathelicidins influence T cell differentiation and DC maturation/function. Cathelicidins can also directly and indirectly promote chemotaxis of other cell types to the site of inflammation.

1.6 Preliminary Data

Preliminary data generated by the laboratory (Lucy Jackson Jones, Emily Gwyer Findlay) suggests that cathelicidin plays a role in the development of Th17 responses.

More specifically, in a pilot study using a model of inflammation induced by heat-killed *Salmonella typhimurium* (HKST), wildtype C57Bl6/JOlaHSd (WT) and cathelicidin knockout (*Camp*^{tm1Rlg}, KO) mice were subcutaneously inoculated with 25 μg HKST in to the top of each hind paw. Mice were culled on day 7 and the draining popliteal lymph nodes removed. Cells were then re-stimulated for three days before assessing cytokine production by ELISA. mCRAMP KO mice displayed a complete loss of IL-17 production and a significant increase in IFNγ compared to WT animals (**Figure 1.10 B**). However, other cytokines measured following re-stimulation, including IL-10, were no different between the two genotypes.

Interestingly, KO mice that were inoculated with *Schistosoma mansoni* eggs, which promotes type-two driven inflammation, did not display a significantly different response: the concentrations of Th2 cytokines, such as IL-5, were no different (**Figure 1.10 C**).

This data shows that mCRAMP-deficient mice have a specific defect in IL-17 production during inflammation. How this occurs or the specific mechanisms behind this defect are not known.

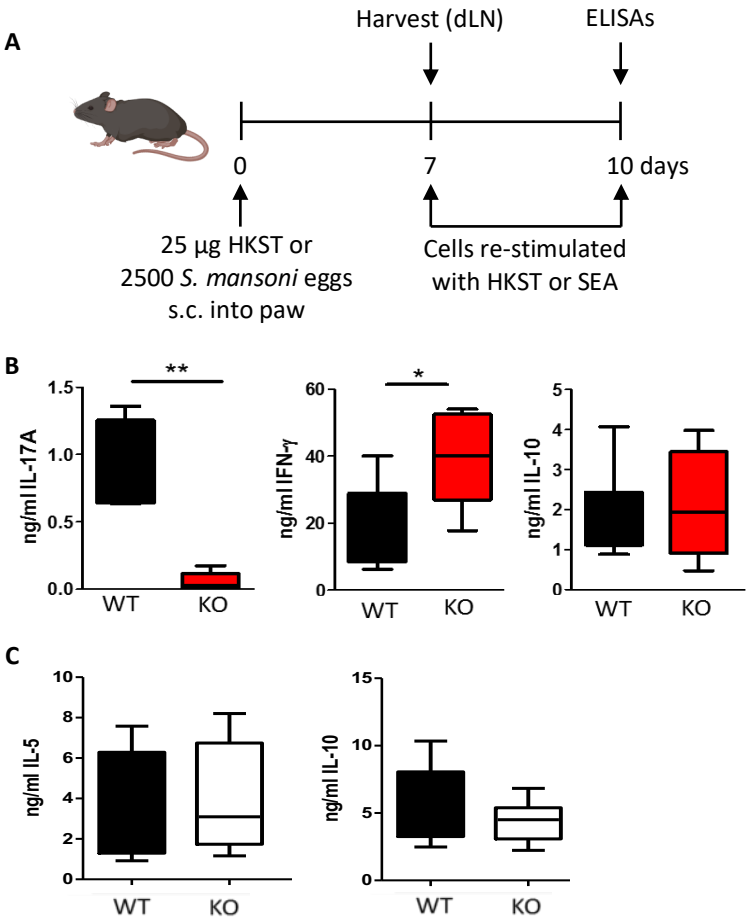


Figure 1.10: mCRAMP KO mice cannot produce IL-17 in response to immunization with heat-killed *S. typhimurium* **A**) WT and CRAMP KO mice were inoculated with 25 µg HKST or 2500 *S.manosni* eggs. Draining popliteal lymph nodes were harvested on day 7 and cells re-stimulated for 3 days before assessing cytokine production by ELISA **B**) Concentration of IL-17A, IFNɣ and IL-10 in cell culture supernatants following re-stimulation with HKST **C**) Concentration of IL-5 and IL-10 in cell culture supernatants following re-stimulation with SEA. Data shown is mean +/- standard error. Statistical significance (where * represents < 0.05, ** < 0.01) was determined using a unpaired t-test. Experiments performed by Lucy Jackson Jones and Emily Gwyer Findlay. WT: wildtype; KO: knockout; HKST: heat-killed *S. typhimurium*; SEA: *S. mansoni* soluble egg antigen; s.c: subcutaneous.

1.7 Aims and Objectives

Preliminary data generated by the laboratory suggests that cathelicidin has a profound impact on T cell behaviour. I hypothesized that cathelicidin plays a role in the generation and amplification of Th17 responses during inflammation. The aims of this project were therefore to:

1) Determine the impacts of mouse cathelicidin (mCRAMP) on $CD4^+$ Th17 and $CD8^+$ Tc17 differentiation *in vitro.*

2) Analyse genetic changes induced by mCRAMP in $CD4^+$ T cells cultured under Th17-driving conditions and identify the mechanism through which it acts.

3) Identify the cellular source of mCRAMP responsible for boosting Th17 responses *in vivo* and establish where these T cells sense the peptide *in vivo*.

CHAPTER 2

Materials and Methods

2.1 Mice

Wild type C57Bl/6JOlaHsd and mCRAMP knockout (*Camp*^{tm1Rlg}) mice were bred and housed in individually ventilated cages, under specific pathogen-free conditions. Knockout mice were backcrossed with the wildtype population for 10 generations. Male and female mice between 6-12 weeks of age were used. All animal experiments were performed in accordance with Home Office UK project licenses PAF438439 and 70/8884, under the Animal (Scientific Procedures) Act 1986.

2.2 Peptides

mCRAMP, LL-37, scrambled LL-37, PP47, human β defensin 2 (hBD2), bactenecin 2A (Bac2A) and indolicidin were custom synthesized by Almac (Penicuik, Scotland) using Fmoc solid phase synthesis and reversed phase HPLC purification (**Table 2.1**). Peptide identity was confirmed by electrospray mass spectrometry. Purity (>95% area) was determined by RP-HPLC and net peptide content determined by amino acid analysis. D-LL-37 was a kind gift from Professor P. Barlow (Edinburgh Napier University). Lyophilized peptides were reconstituted in endotoxin free water at 5 mg/mL. Reconstituted peptides were tested for endotoxin contamination using a Limulus Amebocyte Lysate Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, UK).

Peptide	Amino Acid Sequence	Net Charge
mCRAMP	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ	+6
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	+6
Scrambled LL-37	RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL	+6
D-LL-37	*LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES**	+6
PP47	EFKRIVQRIKDFLRNLVPRTES	+3
Bac2A	RLARIVVIRVAR	+4
Indolicidin	ILPWKWPWWPWRR	+4
hBD2	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP	+6

Table 2.1: **Antimicrobial Peptides**. Peptides used throughout this study, their corresponding amino acid sequences and net charge. *D-enantiomer.

2.3 Mouse Tissue Isolation and Single Cell Preparations

Mice were culled by rising CO_2 and the tissues of interest harvested into complete medium (RPMI, 10% fetal calf serum, 10 units/mL penicillin, 10 μg/mL streptomycin and 2 mM L-glutamine, all supplied by Gibco, ThermoFisher UK).

2.3.1 Spleen, Mesenteric Lymph Nodes and Peyer's Patches

Tissues were mashed through a 100 μM strainer and the resulting cell suspension centrifuged for 5 mins at 900 x g. If necessary, red blood cells were lysed by adding 1 mL of 1X Lysis Buffer (BD, #555899) and incubating at room temperature for 3 mins. The reaction was stopped by adding an excess of 1X PBS and washing the cells as previously described.

2.3.2 Liver

One lobe of the liver was mashed through a 100 μM strainer and the resulting cell suspension centrifuged for 5 mins at 900 x g. Hepatic lymphocytes were isolated by Percoll density gradient centrifugation. Cells were resuspended in 15 mL 37.5% Percoll in HBSS and centrifuged for 10 mins at 900 x g. The supernatant containing hepatocytes was discarded and the cell pellet resuspended in complete medium. Red blood cells were lysed, as previously described.

2.3.3 Lung

One lung lobe was added to 1X PBS containing 0.2 mg/mL Collagenase VIII (Sigma Aldrich, #C2139) and minced with scissors. This was then incubated at 37°C, with shaking, for 20 mins. The reaction was stopped by adding an excess of 1X PBS and mashing the digested tissue through a 100 μM strainer. The cells were washed twice more in 1X PBS, and the red blood cells lysed as previously described.

2.3.4. Bone Marrow

Femurs were taken and the bones cleaned by removing any excess tissue. The bone marrow was then flushed out with complete medium and single cell suspensions prepared by passing the cells through a 19G needle.

2.4 EasySep™ Cell Isolations

2.4.1 Mouse T Cell Isolation

EasySep separation was used to isolate a highly purified population of $CD3^+$ T cells from single-cell suspensions of mouse splenocytes, as per the manufacturer's instructions (StemCell Technologies, #19851). Cells were resuspended at 1 x 10^8 cells/mL in 1 X PBS and incubated with 50 μL/mL rat serum and 50 μl/mL Isolation Cocktail for 10 mins at room temperature. 75 μL/mL Streptavidin RapidSpheres was then added for 2.5 mins. Samples were topped up to 2.5 mL with 1X PBS and placed in a suitable magnet for a further 2.5 mins. Finally, the enriched cell suspension was poured off, ready for use (**Figure 2.1**).

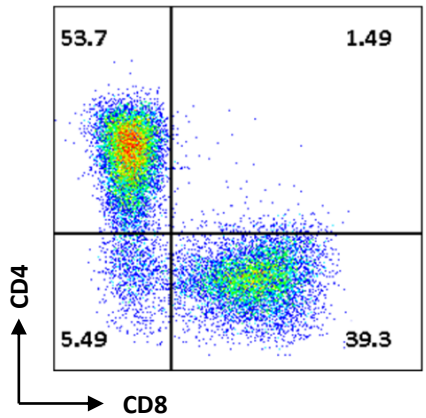


Figure 2.1: Example purity of splenic T cells ($CD4^+$ and $CD8^+$) isolated by EasySep.

2.4.2 Mouse Neutrophil Enrichment

Bone marrow-derived neutrophils were isolated using the EasySep™ Mouse Neutrophil Enrichment Kit, as per the manufacturer's guidelines (StemCell Technologies, #19762). Cells were resuspended at 1 x 10^8 cells/mL in 1X PBS, with 50 μL/mL rat serum and 50 μL/mL Enrichment Cocktail for 15 mins at 4°C. Samples were then washed in 1X PBS and centrifuged for 10 mins, 300 x g. Supernatants were discarded and the cells resuspended in the original volume of 1X PBS. Samples were incubated with 50 μL/mL Biotin Selection Cocktail for another 15 mins at 4°C, followed by 150 μL/mL Magnetic Particles for 10 mins at 4°C. Finally, samples were topped up to 2.5 mL with 1X PBS and placed in a suitable magnet for 3 mins at

room temperature. The enriched cell suspension was then poured off, ready for use (**Figure 2.2**).

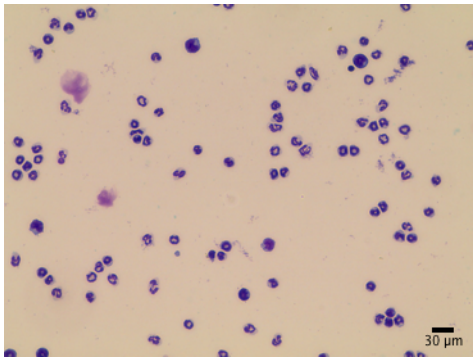


Figure 2.2: Example purity of bone marrow neutrophils isolated by EasySep (magnification: x20).

2.5 *In Vitro* Differentiation of Th1, Th2, Th17 and Treg subtypes

200,000 cells were plated in complete medium and cultured at 37°C, 5% CO_2, 100% humidity, with the correct combination of cytokines and neutralizing antibodies (**Table 2.2**).

2.5.1 Th1 Differentiation

200,000 whole splenic single cell suspensions were plated in an αCD3-coated well in complete medium with rIL-12, rIL-18 and rIL-2, with or without 2.5 µM mCRAMP. Cells were split (1:2) on day 2 and IFNγ production assessed by flow cytometry on day 3.

2.5.2 Th2 Differentiation

200,000 whole splenic single cell suspensions were plated in an αCD3-coated well in complete medium with rIL-4, rIL-2, αIL-12 and αIFNγ, with or without 2.5 µM mCRAMP. Cells were split (1:2) on day 2 or 3, depending on growth. IL-4 production was assessed by flow cytometry on day 4.

2.5.3 Th17 Differentiation

200,000 whole splenic single cell suspensions were plated in an αCD3-coated well in complete medium with rIL-6, rIL-23 and rTGFβ, with or without 2.5 μM mCRAMP. Cells were cultured for 1-3 days before phenotypic analysis by flow cytometry. When using pure populations of $CD3^+$, $CD4^+$ or $CD8^+$ T cells, αCD28 was also included to ensure T cell activation.

2.5.4 Treg Differentiation

$CD4^+$ T cells were isolated from mouse splenocytes by fluorescence activated cell sorting. 200,000 sorted $CD4^+$ T cells were plated in an αCD3-coated well in complete medium with rTGFβ and αCD28, with or without 2.5 μM mCRAMP. FOXP3 expression was assessed by flow cytometry on days 2 and 5.

2.5.5 Splenocyte – neutrophil co-cultures

To examine the effects of neutrophils on Th17 differentiation, splenocyte – neutrophil co-cultures were carried out. Bone marrow neutrophils were isolated by EasySep, as previously described and re-suspended at a concentration of 10 million cells/mL in 1X PBS. Neutrophils were activated and induced to degranulate by stimulating them with 10 μM cytochalasin B for 5 mins at 37°C, with shaking, followed by 100 nM fMLF for a further 25 mins. Neutrophils were plated with whole singe cell splenic suspensions at a ratio of 1:1 and cultured under Th17-driving conditions, as described above.

Cytokine	Company	Catalogue #	Th1	Th2	Th17	Treg
αCD3 (plate-bound)	Biolegend UK	100339	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL
αCD28	Biolegend UK	102115	1 µg/mL	1 µg/mL	1 µg/mL	1 µg/mL
αIL-12	Biolegend UK	505307	-	5 µg/mL	-	-
αIFNγ	Biolegend UK	505812	-	5 µg/mL	-	-
rIL-12	Biolegend UK	577002	25 ng/mL	-	-	-
rIL-18	Gibco	PMC0184	25 ng/mL	-	-	-
rIL-2	Biolegend UK	575402	10 U/mL	40 U/mL	-	-
rIL-4	Biolegend UK	574302	-	4 ng/mL	-	-
rIL-6	Biolegend UK	575706	-	-	20 ng/mL	-
rIL-23	Biolegend UK	589006	-	-	20 ng/mL	-
rTGFβ	Biolegend UK	580706	-	-	3 ng/mL	5 ng/mL

Table 2.2: Cytokines and neutralizing antibodies required for the *in vitro* differentiation of Th1, Th2, Th17 and Treg cells. *αCD28 was added when using $CD3^+$, $CD4^+$ or $CD8^+$ T cells.

2.6 Flow Cytometry

2.6.1 Surface staining for flow cytometry

Cells were harvested and washed twice in 1X PBS before live/dead staining. Samples were incubated for 20 mins with 100 µL of Live/Dead Yellow (in 1X PBS, working dilution: 1/1000; Invitrogen, #L-34959) at 4°C, protected from light. Cells were then washed with 1X PBS, resuspended in 50 µL of FACS buffer (1X PBS + 2% FCS) containing the surface antibody cocktail (**Table 2.3**), and incubated in the dark for 20 mins at 4°C. Samples were washed again and kept at 4°C or fixed in 2% paraformaldehyde.

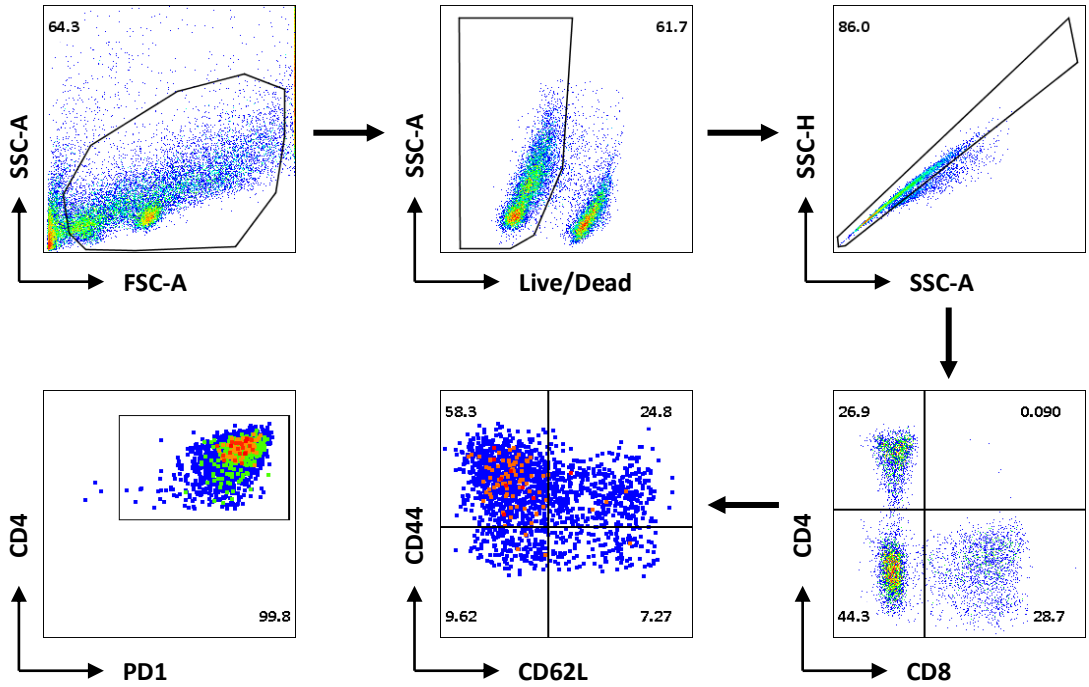


Figure 2.3: **Gating strategy: surface staining of T cells to assess activation status**. Cells were first gated for lymphocytes (SSC-A vs FSC-A). Doublets and dead cells were excluded from the analysis. Surface expression of PD1, CD44 and CD62L was analysed on $CD4^+$ and $CD8^+$ T cells.

2.6.2 Intracellular cytokine staining for flow cytometry

Cells were first stimulated for 4 hrs at 37°C with a Cell Stimulation Cocktail containing PMA, ionomycin and protein transport inhibitors (eBioscience, #00-4970-03, working dilution: 1/500).

After staining for surface markers, cells were fixed and permeabilised for 20 mins at 4°C in 150 µl BD Cytofix/Cytoperm (BD Biosciences, #554722). Cells were then washed with 1X BD Perm/Wash buffer (BD Biosciences, #554723) and stained in 50 µL containing the intracellular cytokine antibody cocktail (**Table 2.3**). Samples were incubated in the dark for 30 mins at 4°C, before being washed again in 1X BD Perm/Wash and resuspended in FACS buffer, ready for analysis.

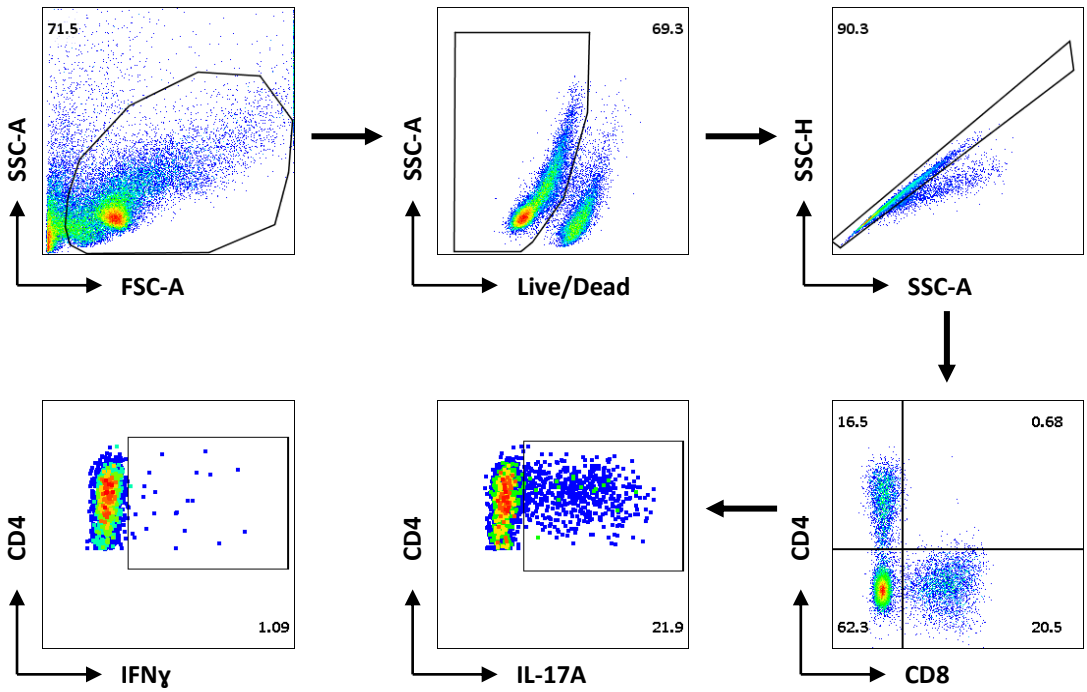


Figure 2.4: **Gating strategy: intracellular staining of T cells to assess cytokine production**. Cells were first gated for lymphocytes (SSC-A vs FSC-A). Doublets and dead cells were excluded from the analysis. IL-17A and IFNγ production by $CD4^+$ and $CD8^+$ T cells was analysed accordingly.

2.6.3 Intracellular transcription factor staining for flow cytometry

Cells were stained for transcription factors using the True-Nuclear Transcription Factor Buffer Set, as per the manufacturer's guidelines (Biolegend, #424401). Cells were harvested and stained for surface markers as previously described, before fixation in 150 µL 1X Fix solution for 1 hour at room temperature. Cells were then washed in 1X Perm Buffer and stained in 50 µL containing the transcription factor antibody cocktail (**Table 2.3**), for 30 mins at room temperature, protected from light. Samples were washed in 1X Perm buffer, resuspended in 1X PBS + 2% FCS and kept at 4°C until ready to run.

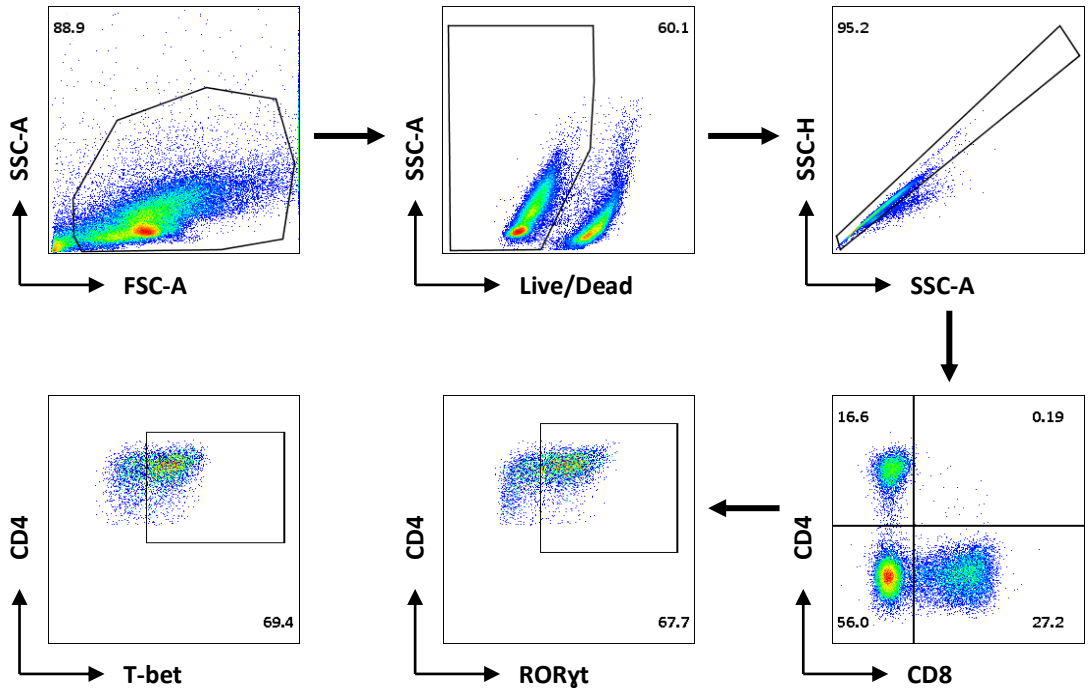


Figure 2.5: **Gating strategy: intracellular staining to assess transcription factor expression**. Cells were first gated for lymphocytes (SSC-A vs FSC-A). Doublets and dead cells were excluded from the analysis. Expression of RORγT and T-bet was analysed on $CD4^+$ and $CD8^+$ T cells.

2.6.4 Annexin/propidium iodide apoptosis assay

Cell viability was assessed by flow cytometry using the Annexin-V-FLUOS Staining Kit, as per the manufacturer's instructions (Roche, #11 858 777 001). Cells were first stained for surface markers before being resuspended in 50 μL Incubation Buffer containing Annexin-V-FLUOS labelling reagent (working dilution: 1/50) and Propidium Iodide Solution (working dilution: 1/50). Cells were incubated for 15 mins at room temperature, protected from light, and then analysed immediately.

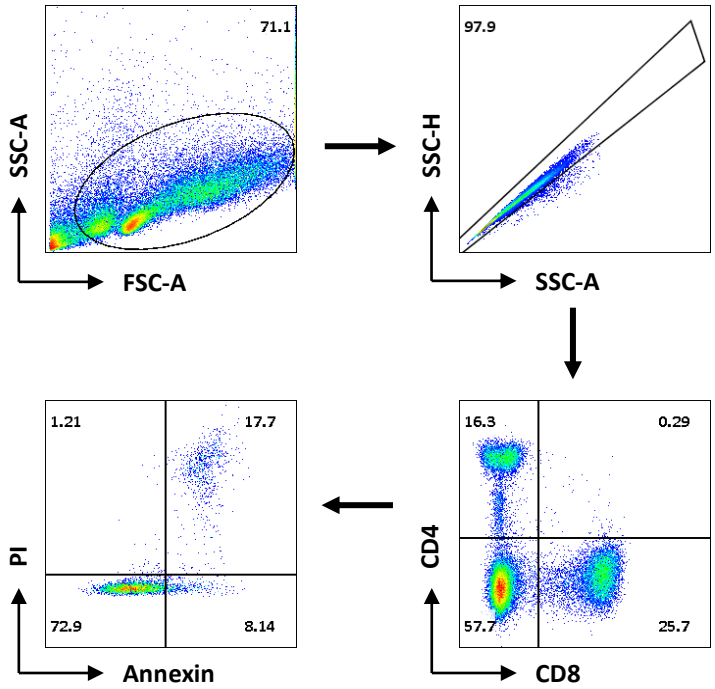


Figure 2.6: **Gating strategy: assessment of T cell viability**. Cells were first gated for lymphocytes (SSC-A vs FSC-A) and doublets excluded from the analysis. Cell viability was assessed by analysing the uptake of propidium iodide (PI) and the expression of annexin by $CD4^+$ and $CD8^+$ T cells.

2.6.5 CFSE cell proliferation assay

In order to assess cellular proliferation, samples were resuspended in 1X PBS and stained with CFSE (carboxyfluorescein succinimidyl ester, Invitrogen, #C34554, working dilution: 1/1000) at 37°C for 20 mins and then washed twice with an excess of media. Proliferation analysis by dye dilution was performed by flow cytometry on day 2, following culture under Th17-driving conditions as previously described (**Figure 2.7**).

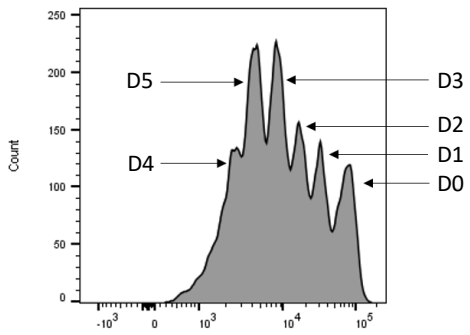


Figure 2.7: **Proliferation analysis by CFSE dye dilution**. Each generation's fluorescence intensity is half that of its parents because the CFSE is split evenly between the daughter cells during mitosis. D: number of divisions.

Marker	Fluorochrome	Clone	Catalogue #	Company	Dilution
AHR	PE-Cy7	4MEJJ	25-5925-80	eBioscience UK	1/200
CD103	BV421	2E7	121422	Biolegend UK	1/200
CD11b	APC-Cy7	M1/70	101226	Biolegend UK	1/200
CD11c	PE-Cy7	N418	117318	Biolegend UK	1/200
CD25	BV510	PC61	102041	Biolegend UK	1/200
CD3	AF700	17A2	100216	Biolegend UK	1/200
CD4	APC	GK1.5	100412	Biolegend UK	1/200
CD4	BUV395	RM4-5	740208	BD Biosciences	1/200
CD4	AF647	GK1.5	100424	Biolegend UK	1/200
CD4	BV785	GK1.5	100453	Biolegend UK	1/200
CD4	FITC	GK1.5	100406	Biolegend UK	1/200
CD44	ef450	IM7	48-0441-82	eBioscience UK	1/200
CD62L	BV570	MEL-14	104433	Biolegend UK	1/200
CD64	APC	X54-5/7.1	139306	Biolegend UK	1/200
CD8	BUV395	53-6.7	563786	Biolegend UK	1/200
CD8	BV650	53-6.7	563234	Biolegend UK	1/200
CD86	BV650	GL-1	105035	Biolegend UK	1/200
CLEC9A	PE	7H11	143504	Biolegend UK	1/200
FOXP3	BV421	MF-14	126419	Biolegend UK	1/200
γδ TCR	APC/Fire 750	GL3	118136	Biolegend UK	1/200
GITR	PerCP-Cy5.5	DTA-1	126315	Biolegend UK	1/200
GMCSF	PE	MP1-22E9	12-7331-82	eBioscience UK	1/100
IFNɣ	PE-Cy7	XMG1.2	505825	Biolegend UK	1/100
IL-17A	PE/Dazzle 594	TC11-18H10.1	506937	Biolegend UK	1/100
IL-17F	AF647	9D3.1C8	517004	Bioegend UK	1/100
IL-22	PerCP-Cy5.5	POLY5164	516411	Biolegend UK	1/100
IL-23R	PE	12B2B64	150904	Biolegend UK	1/200
IL-4	PE	11B11	504103	Biolegend UK	1/100
IL-6Rα	APC	D7715A7	115811	Biolegend UK	1/200
IRF8	APC	V3GYWCH	17-9852-80	eBioscience UK	1/200
MHCII	PerCP-Cy5.5	M5/114.15.2	107625	Biolegend UK	1/200
PD1	PE	29F.1A12	135016	Biolegend UK	1/200
RORɣt	PE	B2D	12-6981-80	eBioscience UK	1/200
SIRPα	FITC	P84	144006	Biolegend UK	1/200
T-bet	PerCP-Cy5.5	4B10	644805	Biolegend UK	1/200
TNFα	BV421	MP6-XT22	506325	Biolegend UK	1/100
XCR1	PerCP-Cy5.5	ZET	148207	Biolegend UK	1/200

Table 2.3: List of antibodies used for flow cytometry.

2.7 Gene Expression

2.7.1 RNA extraction

RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, #74104), as per the manufacturer's guidelines. Up to 1 x 10^6 cells were disrupted in lysis buffer containing β-mercaptoethanol and homogenised using QIAshredder columns. Genomic DNA contamination was eliminated by passing the sample through a gDNA Eliminator spin column. 70% Ethanol was then added to promote selective binding of RNA to the RNeasy membrane. Finally, the sample was washed multiple times and RNA eluted using RNase-free water, pre-warmed to 45°C.

2.7.2 Nanostring

Mouse Th17 cultures were set up as previously described (48 wells per condition, 200,000 cells per well, a total cell count of 9.6 million). On Day 1, wells were pooled and $DAPI^-$ $\gamma\delta^-$ $CD4^+$ T cells were sorted on a BD FACSAria III into 1X PBS + 2% FCS. Samples were centrifuged for 5 mins at 900 x g and the RNA extracted immediately. Multiplex gene expression analysis (Mouse Immunology Panel) was performed by HTPU Microarray Services, University of Edinburgh. Analysis of nanostring data was carried out using nSolver 4.0 and nCounter Advanced Analysis software. Data was normalized to internal reference controls and differentially expressed genes analysed. Genes with a log2 fold change of 1 or more and a p value of 0.05 or less were deemed of interest and statistically significant.

2.8 Protein Expression

2.8.1 Cytokine ELISAs

Concentrations of mouse IL-17A (Biolegend, #432504), IL-2 (Biolegend, #431004), IL-22 (Biolegend, #431004), IL-23 (R&D Systems, #DY1887), IL-6 (Biolegend, #431304), IFNγ (Biolegend, #430804) and TGFβ (R&D Systems, #DY1769) were determined in cell culture supernatants by ELISA, as per the manufacturer's guidelines. 96 well microplates were coated with 100 μL capture antibody and incubated overnight at 4°C. Plates were then

washed and blocked for at least one hour with 1X Assay Diluent. Samples of interest and standards were incubated for 2 hours, prior to the addition of the detection antibody for a further hour. After washing, Streptavidin-HRP was added and incubated for 20 mins, protected from light. Finally, wells were incubated with TMB substrate solution until a strong colour developed, at which point the reaction was stopped (Invitrogen, #11652159). The optical density of each well was read at 450 nm (corrected to 570 nm), and cytokine concentrations calculated from a standard curve.

2.9 *In Vivo* Mouse Models of Inflammation

2.9.1 Heat-killed Salmonella typhimurium model

Heat-killed *Salmonella typhimurium* (HKST) was a kind gift from Professor Andrew McDonald (Lydia Becker Institute, University of Manchester). 25 μg HKST was injected subcutaneously in to the top of each hind paw in 50 μL PBS. Mice were monitored daily and the draining popliteal lymph nodes removed 7 days later. Single cell suspensions were prepared as previously described.

Neutrophil depletion: mice were injected intraperitoneally with 200 μg anti-Ly6G (clone 1A8; BioXCell, #BE0075-1) in 200 μL PBS on days -1, 1 and 3.

2.9.2 Ear inflammation model

Skin inflammation was induced by daily application of a topical dose of 40 mg Aldara™ cream, containing 5% imiquimod, with or without 5 μg CRAMP, on each ear for 3 days. Ears and draining proximal auricular lymph nodes were collected for flow cytometric analysis. Lymph nodes were prepared as previously described. Ears were minced and enzymatically digested in a cocktail containing collagenase VIII (Sigma, #C2139) and DNaseI (Zymo Research, #E1010) for 45 mins at 37°C, with shaking. Samples were dissociated using a gentleMACS™ dissociator, program B (Miltenyi Biotec), passed through a 100 μM filter and washed.

2.10 Statistics

All data shown are expressed as individual data points with line at mean. Analysis was performed with GraphPad Prism software. Two groups were compared with Student's *t*-tests. Multiple groups were compared by one- or two-way analysis of variance tests with either Bonferroni or Dunnett post-tests. "N" refers to individual mice. A minimum of three mice was used over at least 2 experiments to mitigate cage/individual mice effects. A power calculation was performed where necessary. Details of sample sizes are included in all figure legends.

CHAPTER 3

mCRAMP is a Th17 Differentiation Enhancing Factor

3.1 Introduction

Th17 cells play an important role in driving many autoimmune and chronic inflammatory disorders[361]. For instance, IL-23 is essential for inducing the pathogenic features of Th17 lymphocytes and single nucleotide polymorphisms in the *IL23R* gene have been linked to a number of autoimmune conditions, including rheumatoid arthritis (RA), multiple sclerosis (MS), and psoriasis[362,363]. Furthermore, studies using IL-17-, IL-22-, or IL-23-deficient mice, as well as antibody-mediated inhibition, have shown that these Th17-related cytokines are required for the development of autoimmunity[363].

A role for cathelicidin in the pathogenesis of several autoimmune disorders has also recently emerged[364]. For example, levels of human cathelicidin (LL-37) are elevated in the psoriatic epidermis[284]. LL-37 promotes loss of tolerance by forming complexes with inert self-DNA, which stimulates TLR9 in psoriatic dermal plasmacytoid DCs (pDCs) and type I IFN production[334]. Furthermore, increased expression of LL-37 and its activating protease (proteinase-3) have been detected in RA patients[364,365]. LL-37 induces apoptosis of osteoclasts, which contributes to reduced bone formation in arthritic joints[366].

Several reports have linked cathelicidin to IL-17. For instance, elevated levels of LL-37 in hidradenitis suppurativa (HS) lesions positively correlates with the concentration of IL-17 and IL-23[340]. Moreover, the release of type I IFN by pDCs following TLR9 stimulation by LL-37/self-DNA has been shown to promote Th1 and Th17 differentiation in psoriasis[367].

3.2 Aims

Preliminary data generated by the laboratory (Lucy Jackson Jones, Emily Gwyer Findlay) demonstrated that, following inoculation with heat-killed *S. typhimurium* (HKST), mice deficient for cathelicidin (mCRAMP) displayed a complete loss of IL-17 production compared to wildtype animals (**Chapter 1: Figure 10**).

I hypothesized that mCRAMP is necessary for the development of a Th17 response during inflammation. I therefore sought to:

1) Determine whether mCRAMP KO mice possess an underlying T cell defect that is responsible for their inability to produce IL-17 in response to HKST

2) Examine the effects of synthetic mCRAMP on mouse Th17 differentiation *in vitro*

3.3 Results

3.3.1 CD4$^+$ T cells that develop in the absence of mCRAMP have normal cytokine responses

Pilot data demonstrated that mCRAMP KO mice had a near absolute defect in production of IL-17 in response to inoculation with HKST. I therefore first sought to determine whether CD4$^+$ T cells that developed in the absence of this host defence peptide were normal, or whether they possessed an underlying defect that was responsible for their inability to mount a Th17 response.

I compared CD4$^+$ T cell cytokine production *ex vivo* in a variety of different tissues from naïve WT and mCRAMP KO mice by intracellular flow cytometry, in order to assess baseline cytokine responses (**Figure 3.1**). Fluorescence minus one (FMO) controls were used to define gates in the context of data spread due to the use of multiple fluorochromes.

There were no significant differences in the production of IL-17A, IL-17F, IFNγ, IL-22 or GM-CSF in the liver, lungs, spleen, mesenteric lymph nodes or Peyer's patches. However, a significant increase in the frequency of CD4$^+$ TNFα^+ T cells was observed in the liver of mCRAMP-deficient mice (**Figure 3.1 D**: from 46.00% +/- 7.00 to 67.23% +/- 3.00). Despite this, these data suggest that CD4$^+$ T cells that develop in the absence of mCRAMP have relatively normal cytokine responses at resting state, but that the Th17 response is impaired *in vivo* upon stimulation, such as during inflammation induced by HKST.

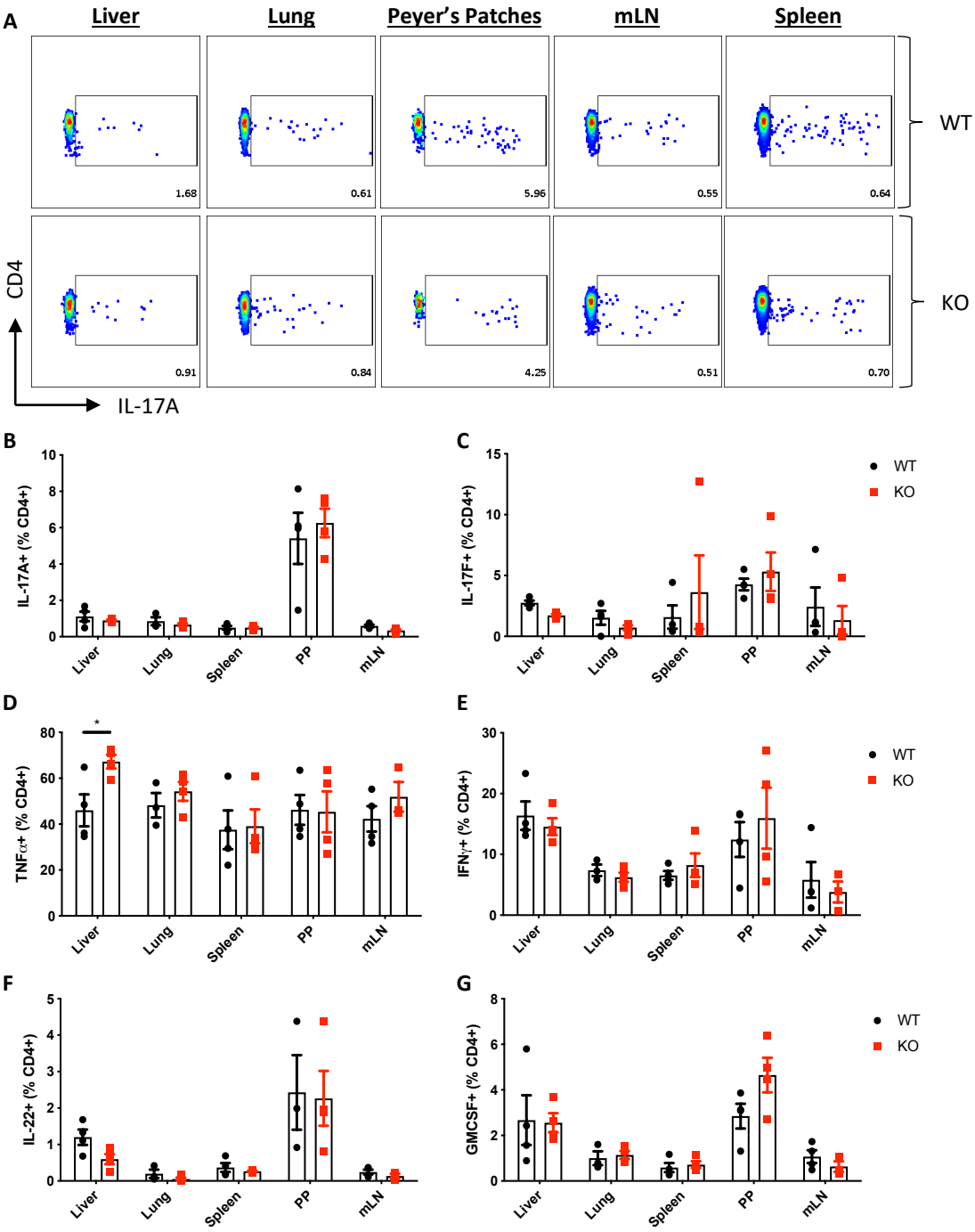


Figure 3.1: Naïve T cells that develop in the absence of mCRAMP have normal cytokine responses. $CD4^+$ T cell cytokine production in the liver, lungs, spleen, Peyer's patches and mesenteric lymph nodes from naïve WT and mCRAMP KO mice was assessed by intracellular flow cytometry **(A)** Representative plots of IL-17A production by $CD4^+$ T cells in WT and mCRAMP KO mice **(B)** Percentages of $CD4^+$ $IL\text{-}17A^+$ T cells in WT and KO mice **(C)** Percentages of $CD4^+$ $IL\text{-}17F^+$ T cells in WT and KO mice **(D)** Percentages of $CD4^+$ $TNF\alpha^+$ T cells in WT and KO mice **(E)** Percentages of $CD4^+$ $IFN\gamma^+$ T cells in WT and KO mice **(F)** Percentages of $CD4^+$ $IL\text{-}22^+$ T cells in WT and KO mice **(G)** Percentages of $CD4^+$ $GM\text{-}CSF^+$ T cells in WT and KO mice. Data shown is mean +/- standard error. N = 4. Statistical significance (where * represents < 0.05) was determined using an unpaired t-test. WT: wild-type; KO: knockout; PP: Peyer's patches; mLN: mesenteric lymph nodes.

3.3.2 CD4⁺ T cells from mCRAMP-deficient mice can produce IL-17 in response to exogenous cytokines

Next, I sought to determine whether CD4⁺ T cells from mCRAMP KO animals were capable of producing IL-17 *in vitro* in response to exogenous Th17-polarizing cytokines. Whole single cell splenic suspensions from WT and mCRAMP KO mice were cultured under Th17-driving conditions with IL-6, IL-23 and TGFβ for 2 days, using the standard Th17-generating protocol[368]. IL-17A production was assessed by intracellular flow cytometry on day 2 (**Figure 3.2**).

There was no significant difference in the percentage of CD4⁺ IL-17A⁺ T cells between WT and KO mice (WT: 14.03% +/- 1.01; KO: 13.40% +/- 1.30), demonstrating that CD4⁺ T lymphocytes from mCRAMP-deficient mice are capable of producing IL-17A and to the same extent as WT T helper cells. This provides further evidence suggesting that CD4⁺ T cells that develop in the absence of mCRAMP are not defective in their capacity to produce IL-17.

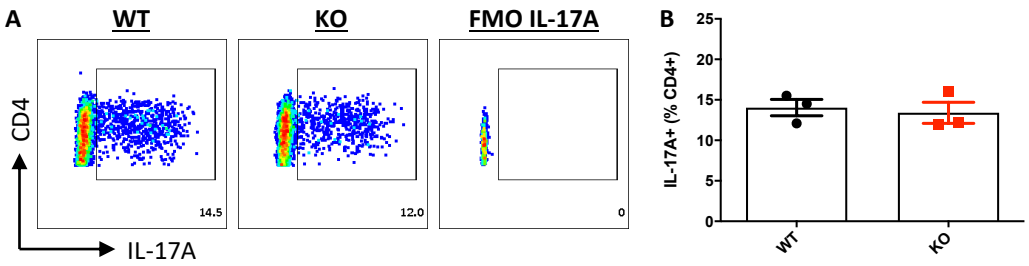


Figure 3.2: CD4⁺ T cells from mCRAMP-deficient mice can produce IL-17A in response to exogenous cytokines. Whole single cell splenic suspensions from WT and KO mice were cultured under Th17-driving conditions (with 20 ng/mL IL-6, 20 ng/mL IL-23 and 3 ng/mL TGFβ) for 2 days **(A)** Representative plots of IL-17A production by CD4⁺ T cells in WT and mCRAMP KO mice on day 2, assessed by flow cytometry **(B)** Percentages of WT and mCRAMP KO CD4⁺ IL-17A⁺ T cells on day 2. Data shown is mean +/- standard error. N = 3. WT: wild-type; KO: knockout.

3.3.3 mCRAMP transiently increases the activation status of CD4⁺ T cells cultured under non-lineage-driving conditions

I have shown that CD4⁺ T lymphocytes that developed in the absence of mCRAMP were capable of producing IL-17 and that cytokine production was unaffected in naïve KO mice. However, preliminary data demonstrated that mCRAMP-deficient animals could not produce IL-17 during inflammation induced by HKST (**Chapter 1: Figure 1.10**). I therefore hypothesised that mCRAMP enhances the development of T cell immunity. To test this, I cultured whole single cell splenic suspensions under non-lineage-driving conditions with plate-bound αCD3, with or without synthetic mouse cathelicidin (mCRAMP).

Figure 3.3 shows that there were no significant differences in the total number of CD4⁺ T lymphocytes between untreated and mCRAMP-treated samples.

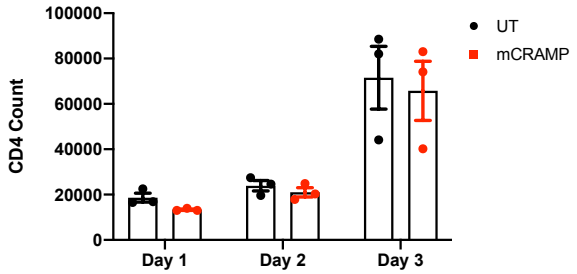


Figure 3.3: mCRAMP has no effect on the total number of CD4⁺ T cells cultured under non-lineage-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under non-lineage-driving conditions (αCD3 only, 5 μg/mL), with or without 2.5 μM synthetic mCRAMP, for 3 days. N = 3.

First, I sought to determine whether mCRAMP had any effect on T cell activation by analysing the expression of classical activation markers including PD1, CD44 and CD62L by flow cytometry every 24 hours for 3 days (**Figure 3.4**). Activated T cells are defined as being $CD62L^-$ $CD44^+$. CD62L acts as a homing receptor for naïve lymphocytes to enter secondary lymphoid tissues where they encounter antigen and is consequently downregulated upon T cell activation, redirecting lymphocytes away from the lymph nodes and towards sites of infection/inflammation[369]. CD44 is another prominent activation marker that also plays a role

in early T cell signalling events[370]. mCRAMP significantly increased the percentage of $CD4^+$ $CD62L^-$ $CD44^+$ T cells, from 47.22% (+/- 3.46) to 53.27% (+/- 2.69), on day 1 (**Figure 3.4 A**).

PD1 is an inhibitory receptor that is involved in the regulation of T cell activation[262]. It is induced by TCR-mediated signalling within 24 hours of stimulation and is therefore not expressed on resting T cells[262]. Almost 100% of $CD4^+$ T cells were $PD1^+$ by day 1 (data not shown). However, Figure 3.4 B shows that mCRAMP significantly increased the geometric mean of PD1, from 8133 (+/- 1040) to 12407 (+/- 948), on day 1. Furthermore, there was a trend indicating that this was also the case on days 2 and 3, although this was not statistically significant.

Taken together, these data suggest that mCRAMP transiently increases the activation status of $CD4^+$ T cells cultured under non-lineage-driving conditions.

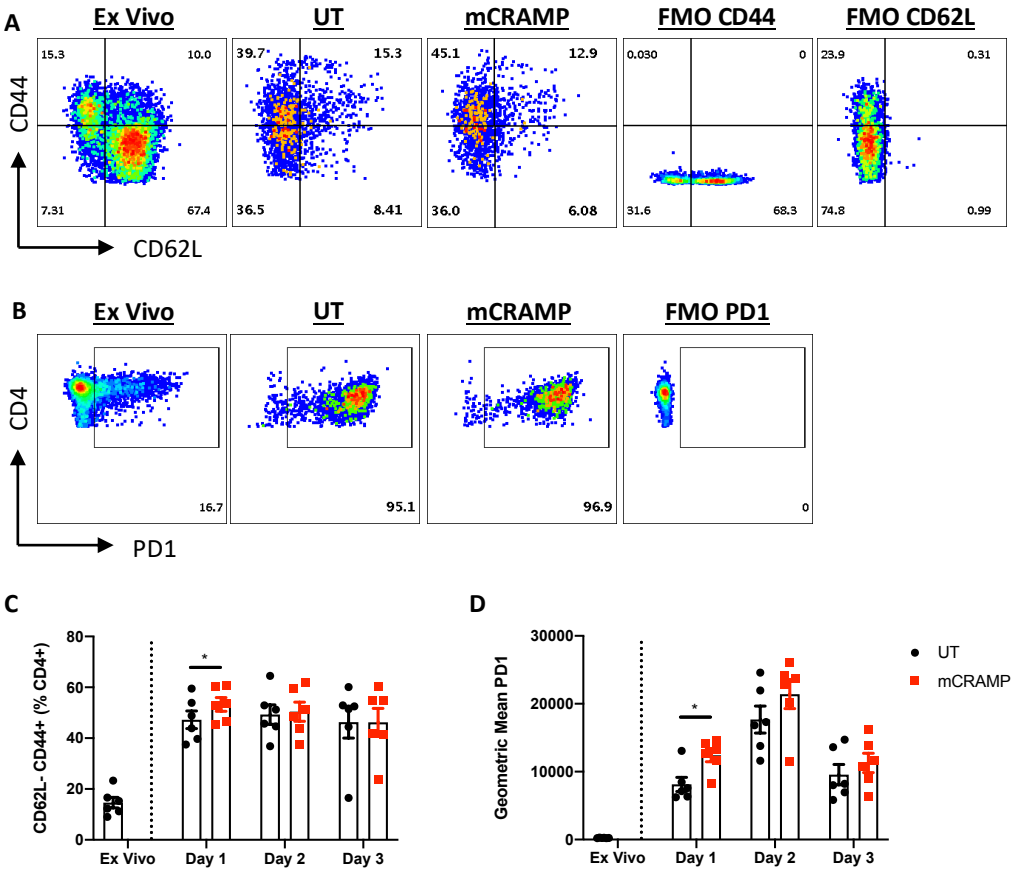


Figure 3.4: mCRAMP transiently increases the activation of CD4⁺ T cells cultured under non-lineage driving conditions. Whole single cell splenic suspensions from WT mice were cultured under non-lineage-driving conditions (αCD3 only, 5 μg/mL), with or without 2.5 μM synthetic mCRAMP, for 3 days **(A)** Representative plots of CD44 and CD62L expression by CD4⁺ T cells on day 1, assessed by flow cytometry **(B)** Representative plots of PD1 expression by CD4⁺ T cells on day 1, assessed by flow cytometry **(C)** Percentages of CD62L⁻ CD44⁺ T cells, *ex vivo* and days 1-3 **(D)** Geometric mean of PD1 expression by CD4⁺ T cells, *ex vivo* and days 1-3. Data shown is mean +/- standard error. N = 6. Statistical significance (where * represents < 0.05) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

3.3.4 mCRAMP does not affect cytokine production by CD4⁺ T cells cultured under non-lineage-driving conditions

Next, I sought to determine whether mCRAMP had any effect on cytokine production. Whole single cell splenic suspensions were cultured with plate-bound αCD3, with or without synthetic mCRAMP, and the expression of IL-17A and IFNγ determined by intracellular flow cytometry (**Figure 3.5**).

The addition of mCRAMP to cells cultured under non-lineage polarizing conditions did not have any effect on CD4⁺ T cell cytokine production: no differences were observed in the percentages of CD4⁺ IL-17A⁺ or CD4⁺ IFNγ⁺ T lymphocytes (**Figure 3.5 B & C**).

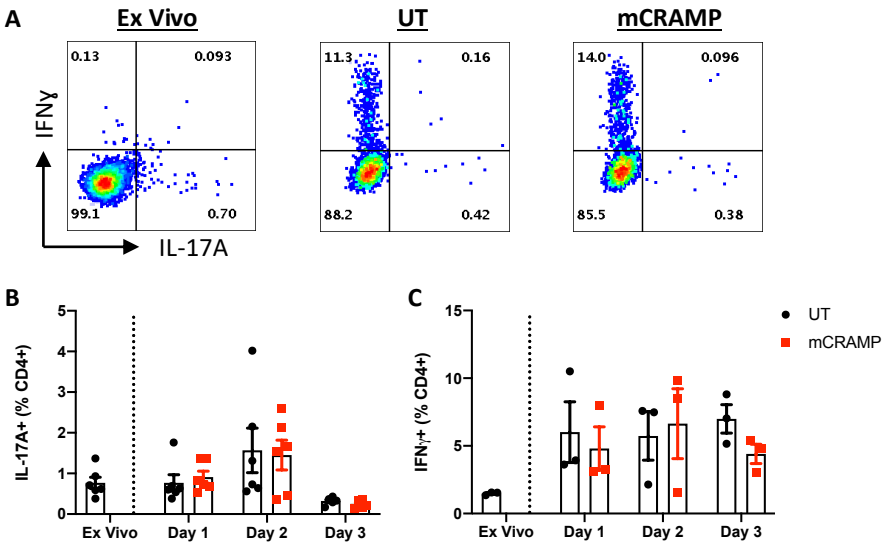


Figure 3.5: mCRAMP does not affect cytokine production by CD4⁺ T cells cultured under non-lineage-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under non-lineage-driving conditions (αCD3 only, 5 μg/mL), with or without 2.5 μM synthetic mCRAMP, for 3 days **(A)** Representative plots of IL-17A and IFNγ expression by CD4⁺ T cells on day 2, assessed by flow cytometry **(B)** Percentages of CD4⁺ IL-17A⁺ T cells, *ex vivo* and days 1-3 **(C)** Percentages of CD4⁺ IFNγ⁺ T cells, *ex vivo* and days 1-3. Data shown is mean +/- standard error. N = 6. Statistical significance (where * represents < 0.05, ** < 0.01, *** < 0.001 and *** < 0.0001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

3.3.5 mCRAMP increases the activation status of CD4⁺ T cells cultured under Th17-driving conditions

mCRAMP transiently increased CD4⁺ T cell activation but had no effect on cytokine production when the cells were cultured under non-lineage-driving conditions in the absence of polarizing cytokines. However, the pilot data discussed previously suggests that this host defence peptide is important for the generation of Th17 responses during inflammation induced by HKST (**Chapter 1: Figure 1.10**). CD4⁺ T cells from mCRAMP-deficient mice were capable of generating normal amounts of IL-17 in response to exogenous IL-17-inducing cytokines. I therefore hypothesised that mCRAMP plays a role in enhancing the development of Th17 lymphocytes. To address this, I examined the effects of adding synthetic mCRAMP to cells cultured under Th17-driving conditions. Whole single cell splenic suspensions were cultured in the presence of IL-6, IL-23 and TGFβ, with or without synthetic mCRAMP.

Figure 3.6 shows that mCRAMP had no significant effect on the total number of CD4⁺ T lymphocytes in Th17 cultures.

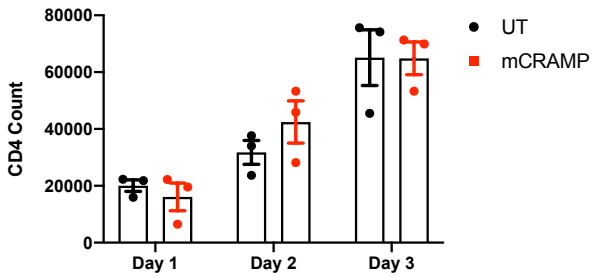


Figure 3.6: mCRAMP has no effect on the total number of CD4⁺ T cells cultured under Th17-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under Th17-driving conditions (20 ng/mL IL-6, 20 ng/mL IL-23 and 3 ng/mL TGFβ), with or without 2.5 μM synthetic mCRAMP, for 3 days. N = 3.

I first sought to determine whether mCRAMP had similar effects on T cell activation when the cells were cultured under Th17-polarizing conditions compared to non-lineage-driving (**Figure 3.7**). Once again, almost 100% of CD4⁺ T cells were PD1⁺ by day 1 (data not shown).

However, the geometric mean of PD1 was significantly increased by mCRAMP on days 1 to 3 (**Figure 3.7 C**). For example, this increased by 43% from 15773 +/- 1134 to 22487 on day 2. Furthermore, activated T cells, defined as $CD62L^{-}$ $CD44^{+}$, were significantly increased following exposure to mCRAMP (**Figure 3.7 D**). This was the case as early as day 1 (UT: 46.27% +/- 3.8; mCRAMP: 57.32% +/- 2.80) but was also observed on days 2 and 3.

These results indicate that mCRAMP increases the activation status of $CD4^{+}$ T cells cultured under Th17-driving conditions. However, unlike when generated in the absence of stimulating cytokines, this is not transient but sustained.

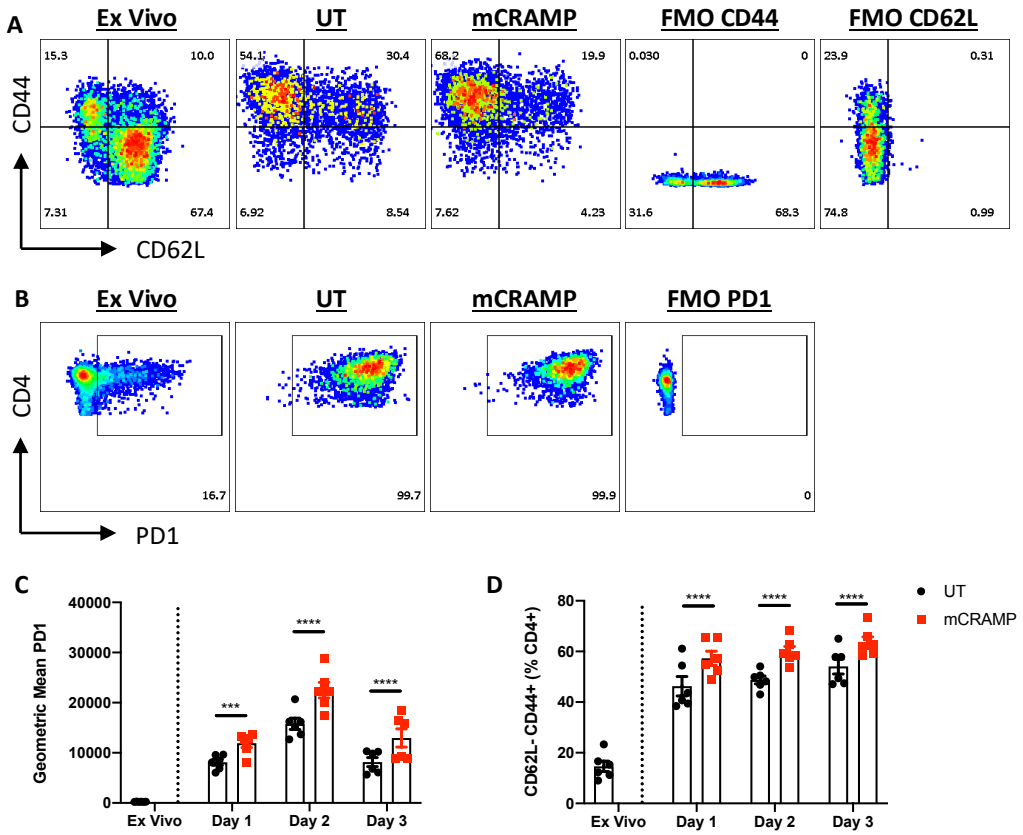


Figure 3.7: mCRAMP increases the activation status of CD4⁺ T cells cultured under Th17-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under Th17-driving conditions, with or without 2.5 μM synthetic mCRAMP, for 3 days **(A)** Representative plots of CD44 and CD62L expression by CD4⁺ T cells on day 2, assessed by flow cytometry **(B)** Representative plots of PD1 expression by CD4⁺ T cells on day 2, assessed by flow cytometry **(C)** Percentages of CD62L⁻ CD44⁺ T cells, *ex vivo* and days 1-3 **(D)** Geometric mean of PD1 expression by CD4⁺ T cells, *ex vivo* and days 1-3. Data shown is mean +/- standard error. N = 6. Statistical significance (where *** represent < 0.001 and **** < 0.0001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

3.3.6 mCRAMP increases IL-17 production by CD4$^+$ T cells cultured under Th17-driving conditions

To further examine the effects of mCRAMP on Th17 cells, I analysed the expression of RORγt and IL-17 by CD4$^+$ T cells. Whole single cell splenic suspensions from WT mice were cultured under Th17-driving conditions, as previously described, with or without synthetic mCRAMP.

RORγt is the master transcription factor that, together with STAT3, promotes the expression of signature Th17 genes in response to IL-6 and TGFβ[43]. To examine Th17 induction, RORγt expression was examined by intracellular flow cytometry every 24 hours for 3 days. Figure 3.8 demonstrates that synthetic mCRAMP induced a significant increase in the percentage of CD4$^+$ RORγt$^+$ T cells on days 1 and 2 (**Figure 3.8 C**). For example, this rose from 17.00 % (+/- 2.62) to 33.93% (+/- 4.97) on day 1.

Reflecting the upregulation of RORγt expression, mCRAMP also significantly increased the percentage of CD4$^+$ IL-17A$^+$ T cells on days 2 and 3 (**Figure 3.8 D & E**). For instance, this rose from 12.48% (+/- 1.10) to 22.15% (+/- 1.35) on day 2 (**Figure 3.8 E**). Interestingly, the geometric mean of IL-17A of CD4$^+$ IL-17A$^+$ T cells was not significantly different between untreated and mCRAMP-treated samples (**Figure 3.8 F**). Moreover, there was a trend suggesting that this was in fact decreased when the cells were cultured in the presence of the peptide (from 3180 +/- 371 to 2882 +/- 270). This could indicate that mCRAMP increased the frequency of CD4$^+$ IL-17A$^+$ T cells but that they were potentially producing less of this cytokine than their untreated counterparts. Nonetheless, the concentration of IL-17A in cell culture supernatants rose significantly, from 1,506 (+/- 338) to 2,073 (+/-476) pg/mL, as measured by ELISA (**Figure 3.7 G**).

These results indicate that synthetic mCRAMP significantly enhances the development of IL-17-producing CD4$^+$ T cells. Furthermore, this increase in response to mCRAMP was concentration-dependent (up until 2.5 μM), as well as time-dependent (**Figure 3.8 C & H**). However, a concentration of 5 μM did not amplify IL-17A production any further, potentially due to the toxic side effects of the peptide and subsequent cell death.

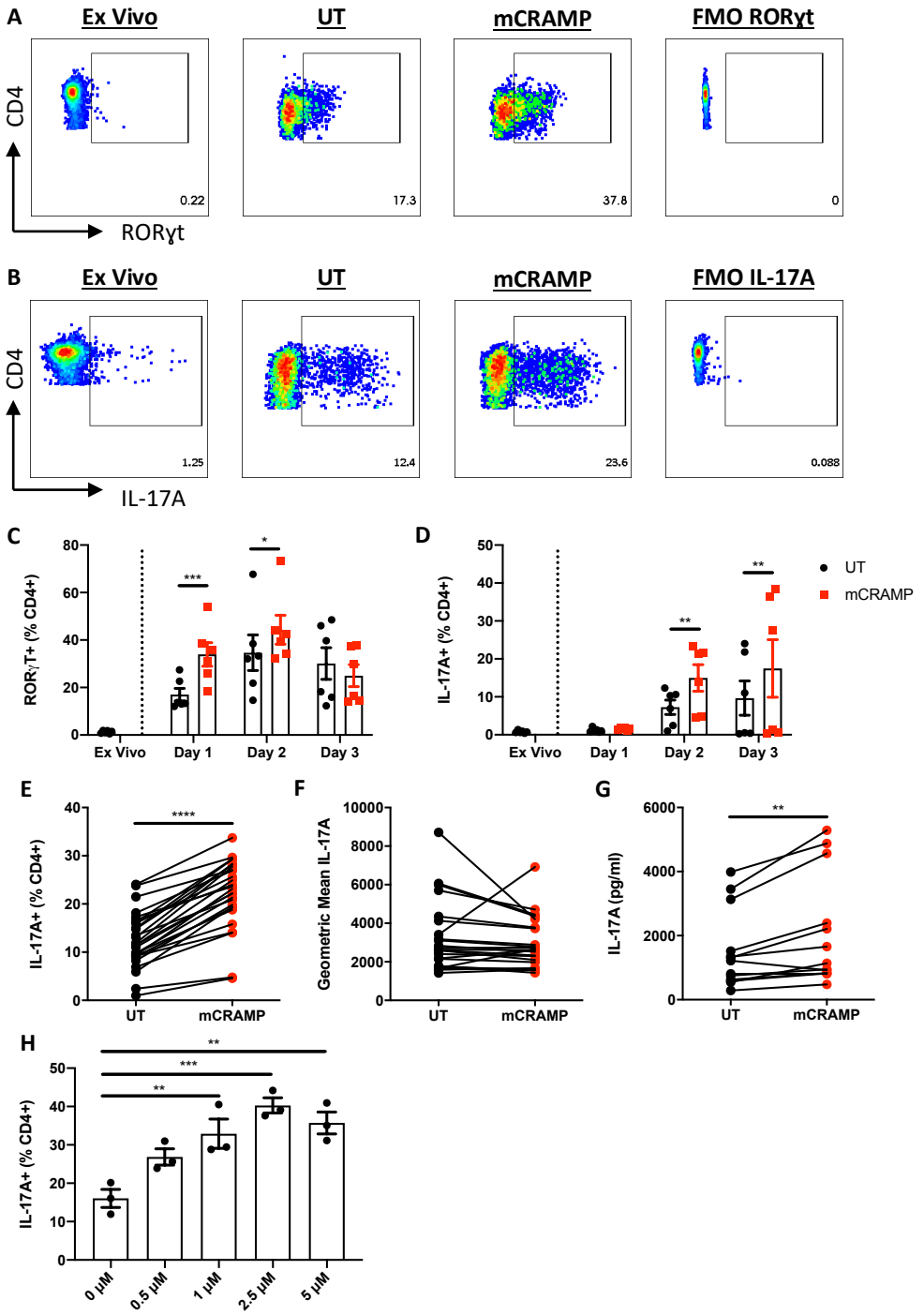


Figure 3.8: mCRAMP increases RORγt expression and enhances the development of IL-17-producing CD4⁺ T cells cultured under Th17-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under Th17-driving conditions, with or without 2.5 μM synthetic mCRAMP, for up to 3 days **(A)** Representative plots RORγt expression by CD4⁺ T cells on day 1, assessed by flow cytometry **(B)** Representative plots of IL-17A production by CD4⁺ T cells on day 2, assessed by flow cytometry **(C)** Percentages of CD4⁺ RORγt⁺ T cells, *ex vivo* and days 1-3 (n = 6) **(D)** Percentages of CD4⁺ IL-17A⁺ T cells, *ex vivo* and days 1-3 (n = 6) **(E)** Percentages of CD4⁺ IL-17A⁺ T cells on day 2 (n = 24) **(F)** Geometric mean of IL-17A expression by CD4⁺ IL-17A⁺ T cells on day 2 (n = 24) **(G)** Concentration of IL-17A in cell culture supernatants on day 2, determined by ELISA (n = 13) **(H)** CD4⁺ IL-17A⁺ vs. mCRAMP dose response (n = 3). Data shown is mean +/- standard error. Statistical significance (where * represents < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test (C & D) or a paired t-test (E & G) or or an ordinary one-way ANOVA with a Dunnett's multiple comparison post-test (H). UT: untreated.

3.3.7 mCRAMP increases IL-17F but not IL-22 production by CD4$^+$ T cells cultured under Th17-driving conditions

Th17 cells express three different dimeric forms of IL-17 of varying potency: IL-17A, IL-17F, and the heterodimer IL-17A/F[98]. I therefore also examined the effects of synthetic mCRAMP on IL-17F production (**Figure 3.9**). Whole single cell splenic suspensions were cultured under Th17-driving conditions, as previously described, with or without synthetic mCRAMP, for 2 days.

mCRAMP significantly increased the percentage of IL-17F single positive CD4$^+$ T cells from 6.67% (+/- 1.37) to 13.26% (+/- 2.20) (**Figure 3.9 C**). Moreover, mCRAMP increased the percentage of CD4$^+$ IL-17A$^+$ IL-17F$^+$ T lymphocytes from 5.37% (+/- 1.19) to 12.68% (+/- 2.41) (**Figure 3.9 D**). The proportion of IL-17A single-positive CD4$^+$ T cells was not significantly different between untreated and mCRAMP-treated samples (**Figure 3.9 B**). However, there was a trend suggesting that mCRAMP also increased the frequency of CD4$^+$ IL-17A$^+$ IL-17F$^-$ T cells; statistical significance was not reached as a result of one particular sample, which was not an obvious outlier and therefore not excluded from analysis (Grubb's test: $P > 0.05$). As a result, these data suggest that the effects of mCRAMP are limited to promoting the differentiation of Th17 cells that express IL-17F and IL-17A/IL-17F dual-producers.

Th17 lymphocytes produce significant quantities of IL-21, which drives the self-amplification phase of Th17 differentiation[26,72]. The concentration of IL-21 in cell culture supernatants measured by ELISA, rose from 37 (+/- 23) to 130 (+/- 80) pg/mL on day 2 following exposure to mCRAMP, although this was not statistically significant due to high variability (**Figure 3.9 E**). Whether or not this rise in IL-21 production is a cause or a consequence of increased Th17 differentiation is unknown.

Th17 lymphocytes can also express IL-22 and CD4$^+$ IL-17$^+$ IL-22$^+$ T cells have been identified as a particularly pathogenic population of autoreactive T lymphocytes during EAE[108,371]. However, mCRAMP did not have any effect on the concentration of IL-22 in cell culture supernatants (**Figure 3.9 F**), indicating that the peptide does not promote the production of all Th17-related cytokines, but has some specificity in its action.

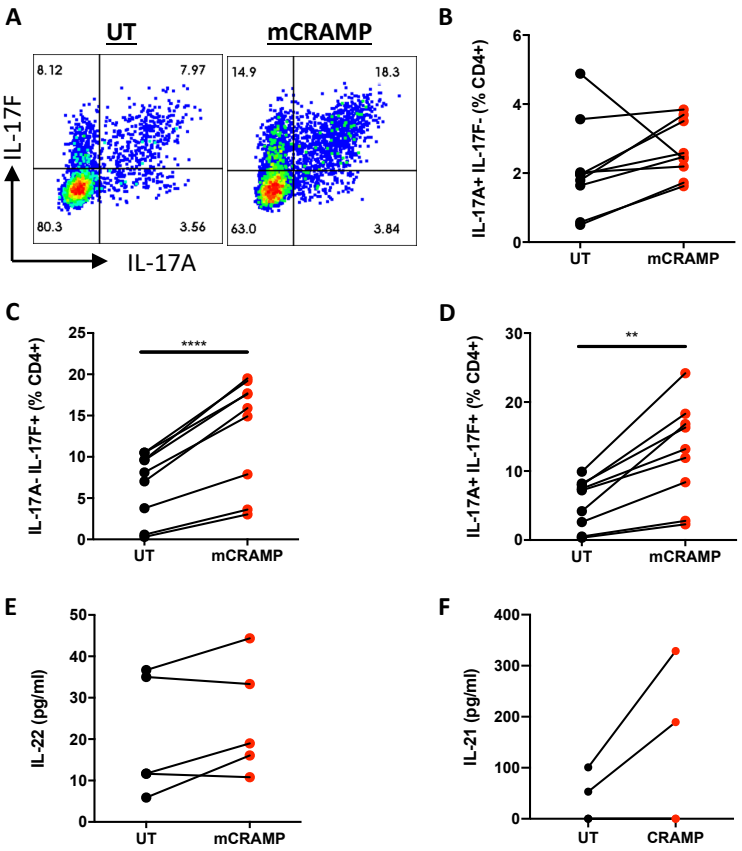


Figure 3.9: mCRAMP increases IL-17F, but not IL-22, production by CD4$^+$ T cells cultured under Th17-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under Th17-driving conditions, with or without 2.5 µM synthetic mCRAMP, for 2 days **(A**) Representative plots of IL-17A and IL-17F expression by CD4$^+$ T cells on day 2, assessed by flow cytometry **(B)** Percentages of CD4$^+$ IL-17A$^+$ IL-17F$^-$ T cells on day 2 (n = 6) **(C)** Percentages of CD4$^+$ IL-17A$^+$ IL-17F$^+$ T cells on day 2 (n = 6) **(D)** Percentages of CD4$^+$ IL-17A$^-$ IL-17F$^+$ T cells on day 2 (n = 6) **(E)** Concentration of IL-21 in cell culture supernatants on day 2, determined by ELISA (n = 4) **(F)** Concentration of IL-22 in cell culture supernatants on day 2, determined by ELISA (n = 5). Data shown is mean +/- standard error. N = 6. Statistical significance (where ** represents < 0.01 and **** < 0.0001) was determined using a paired t-test. UT: untreated.

I have shown that synthetic mCRAMP enhances the development of IL-17-producing CD4$^+$ T lymphocytes *in vitro*. There are several potential mechanisms through which the peptide could act to achieve this (**Figure 3.10**). For example, mCRAMP could act directly on CD4$^+$ T cells, or indirectly, to boost Th17 differentiation. It is also possible that mCRAMP promotes the killing of non-Th17 subsets and/or protects CD4$^+$ IL-17$^+$ T lymphocytes from death, as well as increase the proliferation of these cells.

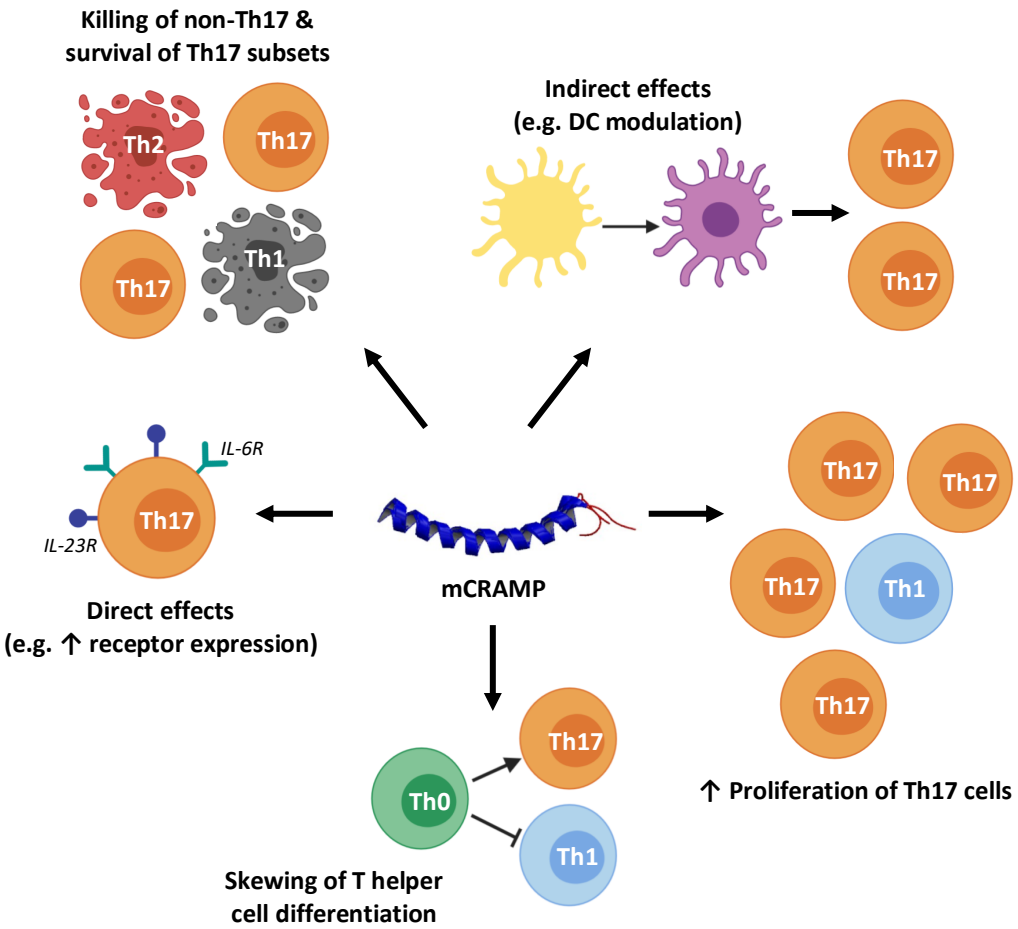


Figure 3.10: **Potential mechanisms of action by mCRAMP to enhance Th17 differentiation**. mCRAMP could act directly on CD4$^+$ T helper cells (e.g. increase the expression of receptors required for Th17 polarization). mCRAMP could also act indirectly, by modulating the function of another cell type, such as dendritic cells. mCRAMP could increase the frequency of CD4$^+$ IL-17$^+$ T cells by increasing their proliferation. mCRAMP could promote the death of non-Th17 subsets and/or protect Th17 lymphocytes from death. DC: dendrtic cell.

3.3.8 mCRAMP acts directly on CD4$^+$ T cells to enhance Th17 differentiation in vitro

The data presented thus far was generated by culturing whole single cell splenic suspensions under Th17-driving conditions. The spleen is predominantly composed of CD4$^+$ (approx. 25%) and CD8$^+$ (approx. 20%) T lymphocytes, as well as B cells (approx. 20%)[372]. However, dendritic cells (approx. 10%), natural killer cells (approx. 5%), macrophages (approx. 5%), and neutrophils (approx. 5%) can also be found[372]. As such, it was unclear whether mCRAMP directly enhances the development of IL-17-producing CD4$^+$ T lymphocytes or acts indirectly via another cell type.

To determine whether mCRAMP could act directly on CD4$^+$ T cells to enhance Th17 differentiation, I sorted CD4$^+$ T lymphocytes by flow cytometry (DAPI$^-$ CD4$^+$ CD8$^-$; purity > 97%) and cultured them under Th17-driving conditions for 2 days, as previously described, with or without synthetic mCRAMP. Figure 3.11 shows that mCRAMP significantly increased the percentage of CD4$^+$ IL-17A$^+$ T cells from 1.18% (+/- 0.03) to 7.29% (+/- 0.02) (**Figure 3.11 B**). This was confirmed by measuring the concentration of IL-17A in cell culture supernatants by ELISA, which rose significantly from 1,518 to 2,550 pg/mL (**Figure 3.11 C**).

The percentage of CD4$^+$ IL-17A$^+$ T cells generated was substantially lower compared to when using whole single cell splenic suspensions. One possible explanation for this is that the quality of activation signals received from DCs within the splenocyte preparations is superior to those provided by αCD3/CD28 antibodies. On the other hand, the fold change observed following exposure to mCRAMP was substantially larger than when culturing total splenocytes (6.18 compared to 1.99). This could potentially be due to the presence of other cell types providing suppressive signals that typically restrain Th17 differentiation. For example, IL-12 is produced mainly by APCs and plays a significant role in promoting Th1 polarization, while simultaneously inhibiting TGFβ-dependent T cell developmental programs (Th17/Treg)[373,374].

Nonetheless, these data suggest that mCRAMP does indeed act directly and specifically on CD4$^+$ T lymphocytes to boost Th17 differentiation.

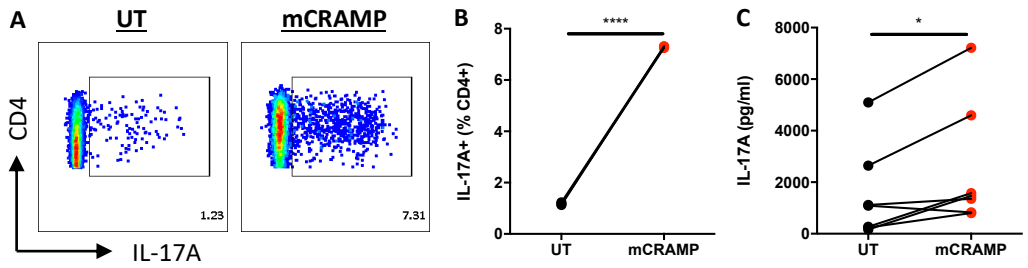


Figure 3.11: mCRAMP acts directly on CD4⁺ T cells. Sorted CD4⁺ T cells were cultured under Th17-driving conditions, with or without 2.5 μM synthetic mCRAMP, for 2 days **(A)** Representative plots of IL-17A production by CD4⁺ T cells on day 2, assessed by flow cytometry **(B)** Percentages of CD4⁺ IL-17A⁺ T cells on day 2 (n = 3) **(C)** Concentration of IL-17A in cell culture supernatants on day 2, determined by ELISA (n = 5). Data shown is mean +/- standard error. Statistical significance (where * represents < 0.05 and **** < 0.0001) was determined using a paired t-test. UT: untreated.

However, it is also possible that mCRAMP acts both directly and indirectly to boost Th17 differentiation, which has an additive effect. For example, human cathelicidin (LL-37) has been shown to modulate the differentiation of immature dendritic cells (DCs) and subsequently promote DC-induced Th1 cell polarization[375]. Furthermore, Gwyer Findlay and colleagues recently demonstrated that exposure of DC precursors to LL-37 dramatically enhances their expansion and differentiation to immature DCs with altered phenotypes, which enhances CD8⁺ T cell proliferation, activation and cytokine production (IFNγ and IL-17A)[338].

To address the possibility that mCRAMP also influenced the behaviour of DCs to indirectly enhance the development of IL-17-producing CD4⁺ T cells, I cultured whole single cell splenic suspensions under Th17-polarizing conditions for 2 days, as previously described, with or without synthetic mCRAMP, and analysed DC phenotype by flow cytometry (**Figure 3.12**). Markers included MHCII and CD86, both DC activation markers that play important roles in T cell activation[8]; XCR1 and CLEC9A, both of which have been shown to play important roles in antigen cross-presentation to CD8⁺ T lymphocytes[376]; CD103, which defines a population of DCs that promote Th17 development[377]. However, there were no significant differences in the proportions of MHCII⁺, SIRPα⁺, XCR1⁺, CD86⁺, CD103⁺ or CLEC9A⁺ DCs between untreated and mCRAMP-treated samples. This suggests that mCRAMP does not act indirectly by influencing the function/phenotype of differentiated DCs.

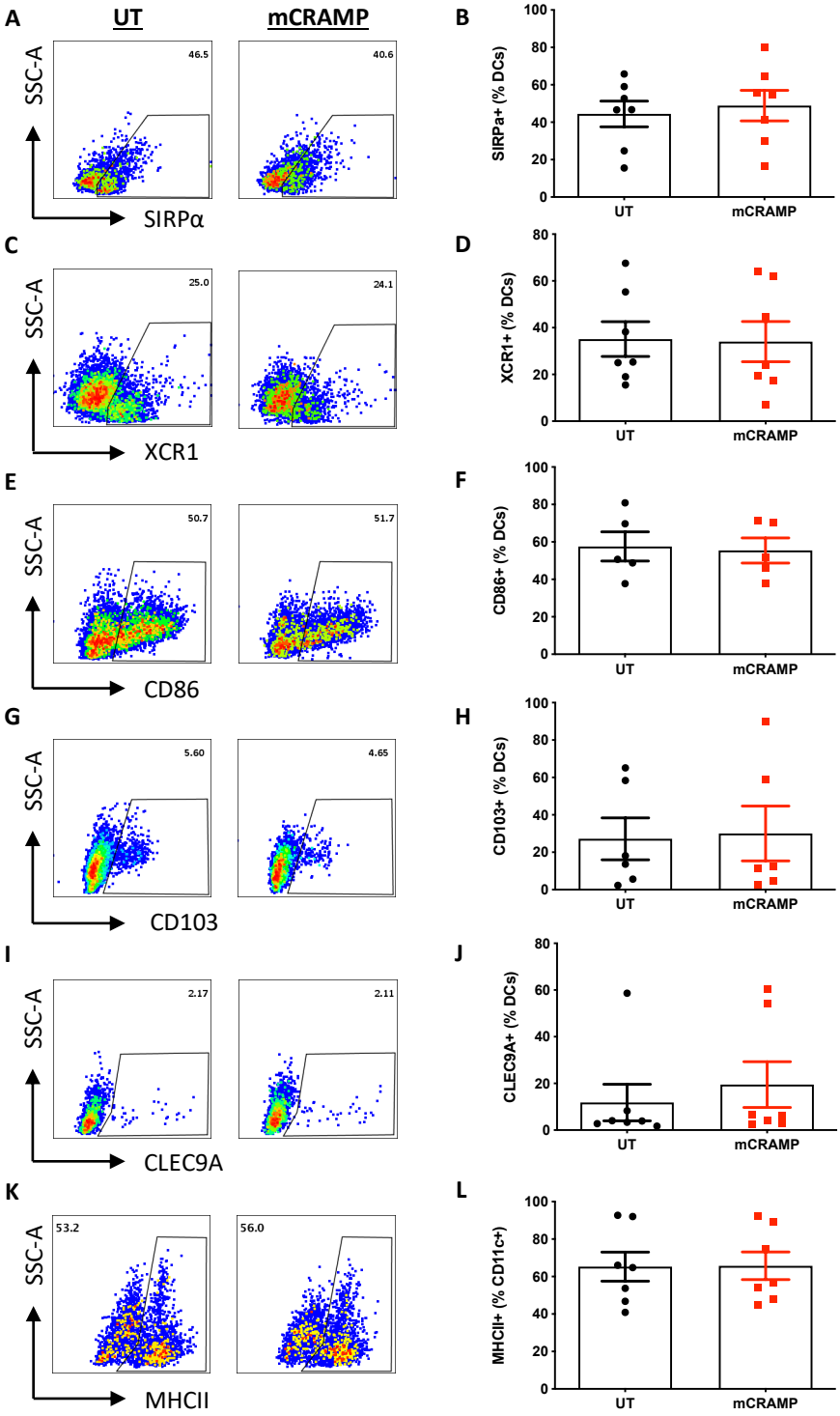


Figure 3.12: mCRAMP does not affect the phenotype of differentiated dendritic cells cultured under Th17-driving conditions. Whole single cell splenic suspensions were cultured under Th17-driving conditions for 2 days, with or without 2.5 μM synthetic mCRAMP **(A)** Representative plots of SIRPα$^+$ DCs **(B)** Percentages of SIRPα$^+$ DCs **(C)** Representative plots of XCR1$^+$ DCs **(D)** Percentages of XCR1$^+$ DCs **(E)** Representative plots of CD86$^+$ DCs **(F)** Percentages of CD86$^+$ DCs **(G)** Representative plots of CD103$^+$ DCs **(H)** Percentages of CD103$^+$ DCs **(I)** Representative plots of CLEC9A$^+$ DCs **(J)** Percentages of CLEC9A$^+$ DCs **(K)** Representative plots of MHCII$^+$ cells (gated on CD11c+) **(L)** Percentages of MHCII$^+$ cells (gated on CD11c$^+$). Data shown is mean +/- standard error. N = 6. UT: untreated.

3.3.9 mCRAMP has no significant effect on CD4$^+$ T cell expression of IL-6R or IL-23R

I have shown that mCRAMP can act directly on CD4$^+$ T cells to enhance the development of IL-17-producing lymphocytes but how the peptide achieves this is unclear. One possibility is that mCRAMP upregulates the expression of receptors required for Th17 differentiation, thereby increasing their sensitivity to Th17-polarizing cytokines. I therefore analysed the expression of IL-6R and IL-23R by CD4$^+$ T cells when cultured under Th17-driving conditions, with or without synthetic mCRAMP (**Figure 3.13**). The lack of a reliable antibody that can be used to detect TGFβR by flow cytometry prevented the analysis of CD4$^+$ TGFβR$^+$ T cells.

Figure 3.13 shows that the results were highly variable but no significant differences were observed in the frequencies of CD4$^+$ IL-6R$^+$ or IL-23R$^+$ T cells between untreated and mCRAMP-treated samples. This suggests that this is not how the peptide enhances Th17 differentiation *in vitro*.

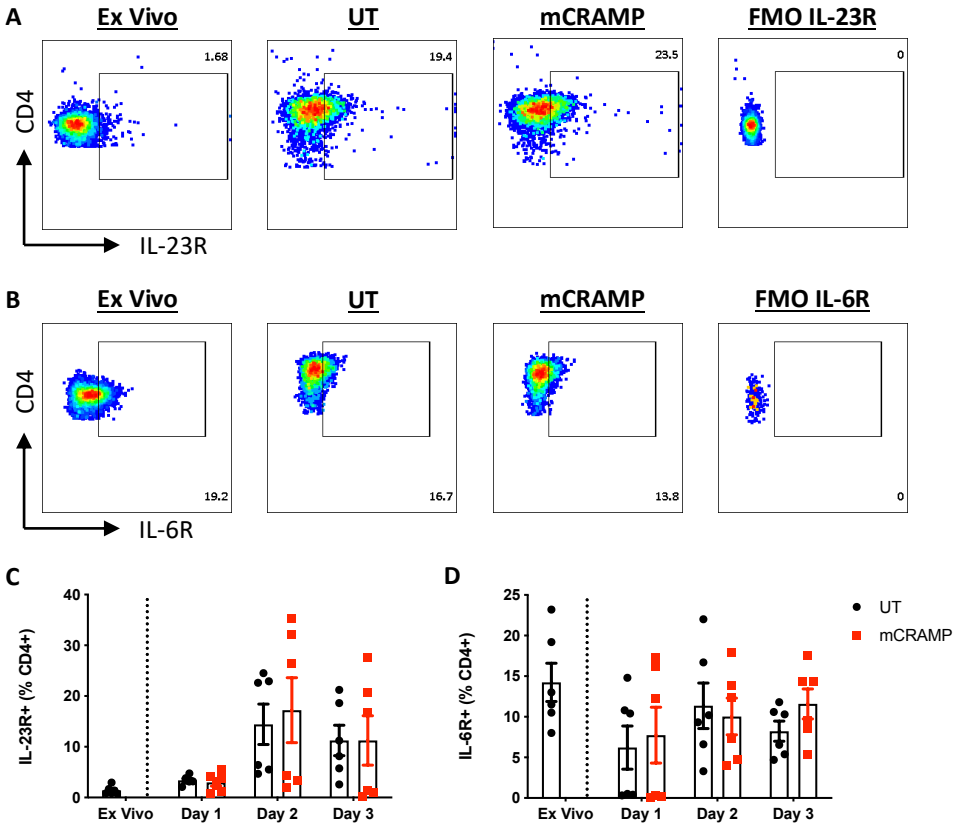


Figure 3.13: mCRAMP has no significant effect on IL-23R or IL-6R expression by CD4⁺ T cells cultured under Th17-driving conditions. Whole single cell splenic suspensions were cultured under Th17-driving conditions, with or without 2.5 μM synthetic mCRAMP, for up to 3 days **(A)** Representative plots of IL-23R expression by CD4⁺ T cells on day 2, assessed by flow cytometry **(B)** Representative plots of IL-6R expression by CD4⁺ T cells on day 2, assessed by flow cytometry **(C)** Percentages of CD4⁺ IL-23R⁺ T cell, *ex vivo* and days 1-3 **(D)** Percentages of CD4⁺ IL-6R⁺ T cell, *ex vivo* and days 1-3. Data shown is mean +/- standard error. N = 6. UT: untreated.

3.3.10 mCRAMP increases the viability of CD4$^+$ T cells

Another potential mechanism through which mCRAMP could enhance Th17 differentiation is by promoting the survival of CD4$^+$ IL-17$^+$ T cells and the death of non-Th17 subsets. Indeed, mCRAMP has previously been shown to induce programmed cell death in certain T cell subsets. For example, Mader and colleagues demonstrated that human cathelicidin (LL-37) induces granzyme-mediated apoptosis in cytotoxic CD8$^+$ and regulatory T cells[360,378].

I therefore performed an annexin/propidium iodide (PI) apoptosis assay to examine cell death in non-lineage-driving and Th17 cultures, treated with synthetic mCRAMP. Annexin V is a Ca^{2+}-dependent phospholipid binding protein that has high affinity for phosphatidylserine (PS)[379]. PS locates to the cytoplasmic surface of the cell membrane in live cells but is translocated to the outer leaflet during apoptosis, thereby exposing it[379]. PI is a dead cell marker that binds to nucleic acids but cannot permeate living cells[380]. Together, annexin and PI can be used to distinguish living, apoptotic and necrotic cells (**Figure 3.15 A**).

Unfortunately, this technique cannot be employed alongside intracellular cytokine staining due to the fixation step. Various studies have identified Th17 cells based on their expression of several surface markers[381,382]. For example, Annunziato et al. demonstrated that Th17 lymphocytes selectively express CCR6[381]. However, the definition of Th17 cells by surface markers might overestimate their frequency in comparison to functional assessment of IL-17 production by intracellular cytokine staining, although both methods have been suggested to yield proportionate results[383]. In my hands, I found CCR6 to be inadequate for the identification of Th17 lymphocytes: I saw no CD4$^+$ CCR6$^+$ IL-17A$^+$ T cells and while the percentage of CD4$^+$ IL-17A$^+$ T cells was increased following exposure to mCRAMP, the frequency of CD4$^+$ CCR6$^+$ T lymphocytes was decreased (**Figure 3.14**). As a result, it was only possible to analyse the CD4$^+$ T cell population as a whole using this method.

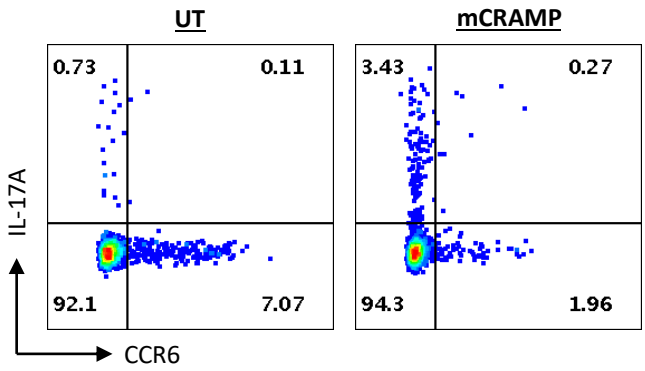


Figure 3.14: CCR6 is not a suitable surface marker for the identification of CD4$^+$ IL-17$^+$ T cells. Whole single cell splenic suspensions from WT mice were cultured under Th17-driving conditions, with or without 2.5 μM synthetic mCRAMP. Cells were stained on day 2 for CCR6 and intracellular IL-17A. Very few cells were CCR6/IL-17A double-positive. CRAMP appeared to decrease the percentage of CD4$^+$ CCR6$^+$ T lymphocytes but increase the percentage of IL-17-single positive cells. UT: untreated.

mCRAMP had no effect on the total number of CD4$^+$ T cells following culture under non-lineage-polarising conditions, (**Figure 3.15 B**) but significantly increased the percentage of CD4$^+$ annexin$^-$ PI$^-$ ("alive") T lymphocytes on day 3, from 43.13% (+/- 6.90) to 53.70% (+/- 3.51) (**Figure 3.15 D**). Furthermore, there was a trend suggesting mCRAMP decreased the proportion of CD4$^+$ annexin$^+$ PI$^+$ ("necrotic") T cells, although this was not statistically significant (**Figure 3.15 H**). No differences in the percentage of apoptotic (annexin$^+$ PI$^-$) T cells were observed between untreated and mCRAMP-treated samples (**Figure 3.15 F**).

When the cells were cultured under Th17-driving conditions, mCRAMP had no effect on the total number of CD4$^+$ T cells (**Figure 3.15 C**) but significantly increased the percentage of CD4$^+$ annexin$^-$ PI$^-$ (**Figure 3.15 E**) and decreased the percentage of CD4$^+$ annexin$^+$ PI$^+$ T cells (**Figure 3.15 I**) on days 2 to 3 and 1 to 3, respectively. For instance, on day 3, mCRAMP increased the proportion of CD4$^+$ annexin$^-$ PI$^-$ T cells from 57.32% (+/- 6.00) to 70.18% (+/- 1.49), and decreased CD4$^+$ annexin$^+$ PI$^+$ from 28.43% (+/- 5.15) to 15.98% (+/- 1.84). No significant differences in the frequency of CD4$^+$ annexin$^+$ PI$^-$ T cells were observed between untreated and mCRAMP-treated samples (**Figure 3.15 G**).

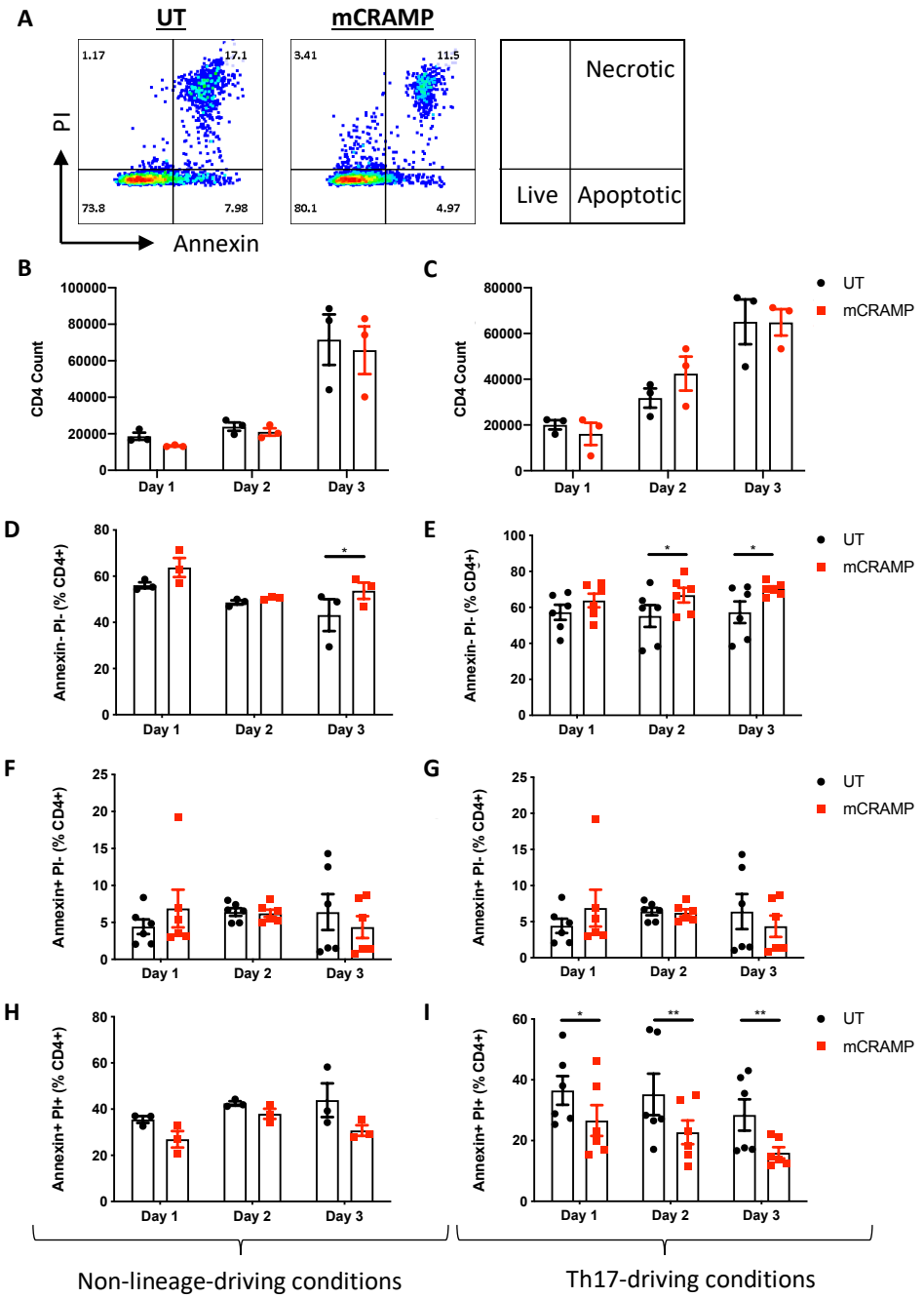


Figure 3.15: mCRAMP increases CD4⁺ T cell viability. Whole single cell splenic suspensions were cultured under non-lineage-driving or Th17-polarizing conditions, with or without 2.5 µM mCRAMP, for up to 3 days **(A)** Representative plots of annexin/PI staining of CD4⁺ T cells on day 2, assessed by flow cytometry **(B)** Total numbers of CD4⁺ T cells in non-lineage driving cultures **(C)** Total numbers of CD4⁺ T cells in Th17 cultures **(D)** Percentages of CD4⁺ annexin⁻ PI⁻ T cells on days 1-3 following culture under non-lineage-driving conditions (n = 3) **(E)** Percentages of CD4⁺ annexin⁻ PI⁻ T cells on days 1-3 following culture under Th17-driving conditions (n = 6) **(F)** Percentages of CD4⁺ annexin⁺ PI⁻ T cells on days 1-3 following culture under non-lineage-driving conditions (n = 3) **(G)** Percentages of CD4⁺ annexin⁺ PI⁻ T cells on days 1-3 following culture under Th17-driving conditions (n = 6) **(H)** Percentages of CD4⁺ annexin⁺ PI⁺ T cells on days 1-3 following culture under non-lineage-driving conditions (n = 3) **(I)** Percentages of CD4⁺ annexin⁺ PI⁺ T cells on days 1-3 following culture under Th17-driving conditions (n = 6). Data shown is mean +/- standard error. Statistical significance (where * represents < 0.05 and ** < 0.01) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

To identify the effects of mCRAMP on the viability of $CD4^+$ $IL\text{-}17^+$ and $CD4^+$ $IL\text{-}17^-$ T lymphocytes, I analysed the uptake of a fixable live/dead cell stain on day 2 in Th17 cultures (**Figure 3.16**). Using this method, I observed a significant decrease, from 39.08% (+/- 2.68) to 26.65% (+/- 3.02), in the percentage of dead $CD4^+$ T cells (**Figure 3.16 A & B**). This supports the results obtained from the annexin/PI apoptosis assay and suggests that mCRAMP protects the $CD4^+$ T cell population as a whole from death. However, it is important to note that this could reflect an increase in the viability of a particular subset (within the whole $CD4^+$ population).

mCRAMP significantly decreased the percentage of dead $CD4^+$ $IL\text{-}17^+$ T lymphocytes from 19.71% (+/- 2.32) to 12.13% (+/- 1.83) (**Figure 3.16 C & D**). Furthermore, the percentage of dead $CD4^+$ $IL\text{-}17^-$ T cells was also significantly reduced (41.10% +/- 2.86 to 29.57% +/- 3.44) (**Figure 3.16 E & F**).

These results imply that mCRAMP acts as a survival factor to increase the viability of all $CD4^+$ T cells cultured under Th17-driving conditions. This does not therefore explain the increase in the percentage of $CD4^+$ $IL\text{-}17^+$ T cells induced by the peptide.

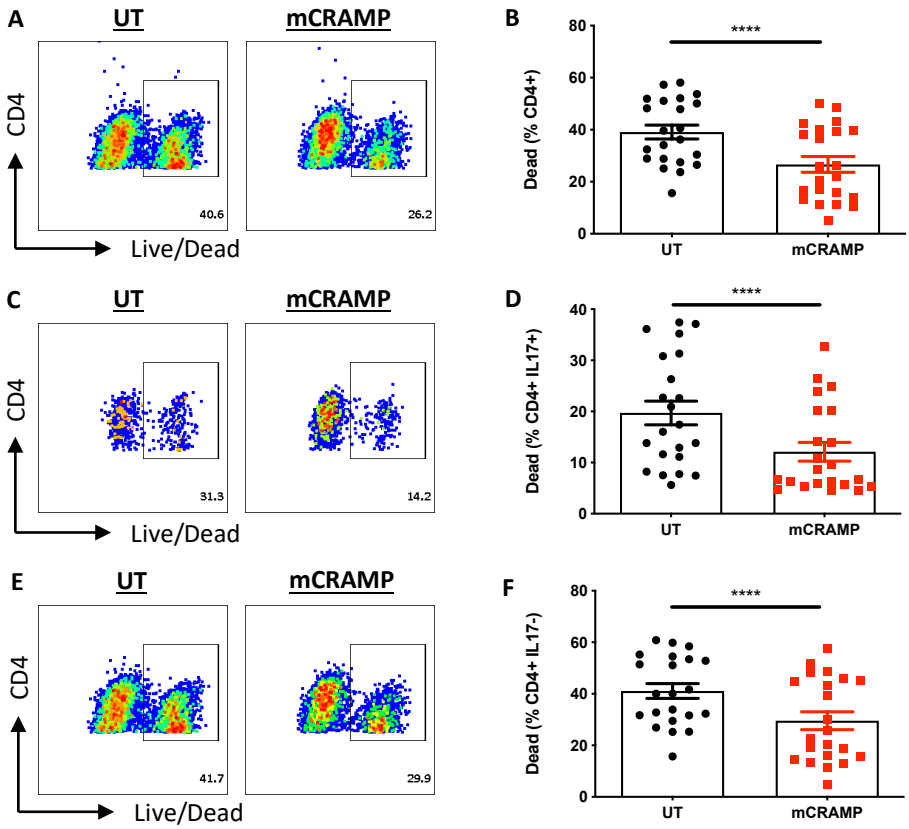


Figure 3.16: mCRAMP promotes the survival of $CD4^+$ $IL\text{-}17^+$ and $IL\text{-}17^-$ T cells. Whole single cell splenic suspensions were cultured under Th17-driving conditions, with or without 2.5 µM mCRAMP, for 2 days **(A)** Representative plots of dead $CD4^+$ T cells on day 2 (gated on single, $CD4^+$ lymphocytes), assessed by flow cytometry **(B)** Percentages of dead $CD4^+$ T cells on day 2 **(C)** Representative plots of dead $CD4^+$ $IL\text{-}17A^+$ T cells on day 2 (gated on $CD4^+$ $IL\text{-}17A^+$), assessed by flow cytometry **(D)** Percentages of dead $CD4^+$ $IL\text{-}17A^+$ T cells on day 2 **(E)** Representative plots of dead $CD4^+$ $IL\text{-}17A^-$ T cells on day 2 (gated on $CD4^+$ $IL\text{-}17A^-$), assessed by flow cytometry **(F)** Percentages of dead $CD4^+$ $IL\text{-}17A^-$ T cells on day 2. Data shown is mean +/- standard error. N = 22. Statistical significance (where **** represents < 0.0001) was determined using a paired t-test. UT: untreated.

3.3.11 mCRAMP does not increase the proliferation of CD4⁺ T cells cultured under Th17-driving conditions

To determine whether mCRAMP enhanced Th17 differentiation by specifically increasing the proliferation of $CD4^+$ $IL\text{-}17^+$ T lymphocytes, I monitored the proliferation of these cells by CFSE dye dilution (**Figure 3.17**). CFSE is a non-fluorescent dye that passively diffuses across cell membranes[384]. Cleavage by intracellular esterases within viable cells renders it highly fluorescent and capable of covalently binding to protein amine groups via its succinimidyl ester group[384]. As cells divide, the dye is distributed uniformly between daughter cells, allowing for the analysis of proliferation by serial halving of fluorescence intensity[384].

mCRAMP had no effect on the proliferation of total $CD4^+$ T cells when cultured under Th17-driving conditions (**Figure 3.17 A & B**).

Due to high levels of cell death observed in stimulated, intracellular cytokine- and CFSE-stained cultures, I analysed the division of $ROR\gamma t^+$ and $ROR\gamma t^-$ lymphocytes in order to monitor Th17 proliferation. The proliferation of $CD4^+$ $ROR\gamma t^+$ T cells was no different between untreated and mCRAMP-treated samples (**Figure 3.17 C & D**), which suggests that the peptide does not boost Th17 differentiation by increasing the proliferation of this T helper cell subset. Furthermore, there were no significant differences in the proliferation of $CD4^+$ $ROR\gamma t^-$ T cells (**Figure 3.17 E & F**), indicating that mCRAMP does not suppress the proliferation of non-IL-17-producing lymphocytes either.

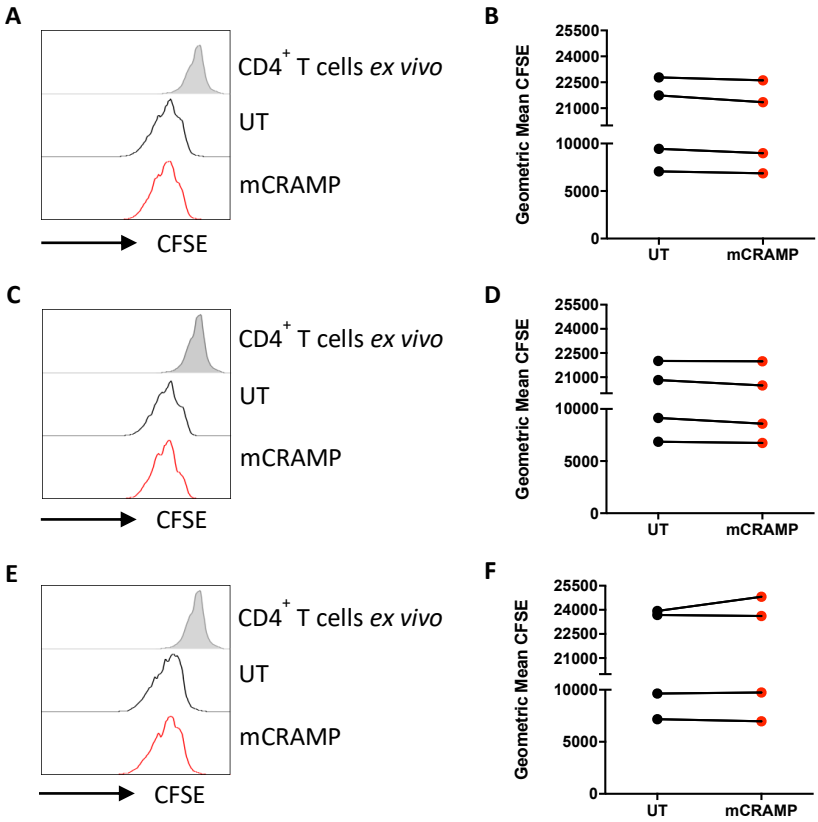


Figure 3.17: mCRAMP has no effect on CD4⁺ T cell proliferation. CFSE-stained whole single cell splenic suspensions were cultured under Th17-driving conditions, with or without 2.5 µM synthetic mCRAMP, for 2 days **(A)** Representative CFSE plot of CD4⁺ T cells, assessed by flow cytometry **(B)** Geometric mean of CFSE by CD4⁺ T cells **(C)** Representative CFSE plot of CD4⁺ RORɣt⁺ T cells, assessed by flow cytometry **(D)** Geometric mean of CFSE by CD4⁺ RORɣt⁺ T cells **(E)** Representative CFSE plot of CD4⁺ RORɣt⁻ T cells, assessed by flow cytometry **(F)** Geometric mean of CFSE by CD4⁺ RORɣt⁻ T cells. Data shown is mean +/- standard error. N = 4. UT: untreated.

3.3.12 mCRAMP acts specifically on the Th17 pathway

I have shown that mCRAMP enhanced the development of IL-17-producing $CD4^+$ T cells when cultured under Th17-driving conditions *in vitro*. To determine whether this host defence peptide acts specifically on the Th17 pathway, or non-specifically boosts all cytokine production, I next assessed the effects of mCRAMP on the differentiation of other T helper subsets.

Whole single cell splenic suspensions were cultured under Th1- (IL-12, IL-18, IL-2) or Th2-polarizing (IL-4, IL-2, αIL-12, αIFNγ) conditions, with or without synthetic mCRAMP. Due to poor polarization using splenocytes, $CD4^+$ T cells isolated by EasySep were cultured under Treg-driving conditions (TGFβ), with or without synthetic mCRAMP. The fold change in the percentage of $CD4^+$ $IFN\gamma^+$ (Th1), $CD4^+$ $IL\text{-}4^+$ (Th2) or $CD4^+$ $FOXP3^+$ (Treg) T cells was determined by intracellular flow cytometry (**Figure 3.18**).

Whilst mCRAMP induced a 1.99-fold increase in the percentage of $CD4^+$ $IL\text{-}17A^+$ T cells, it did not have any effect on Th1 or Th2 differentiation: no significant changes in the proportions of $CD4^+$ $IFN\gamma^+$ or $IL\text{-}4^+$ lymphocytes were detected. This is in accordance with the preliminary data demonstrating that cytokine production following inoculation with *Schistosoma mansoni* eggs, which induces a type 2 immune response, was not significantly different in mCRAMP-deficient mice (**Chapter 1: Figure 1.10**).

However, a small (1.30 fold) increase in the percentage of $CD4^+$ $FOXP3^+$ cells was observed on day 2 when $CD4^+$ T lymphocytes were cultured under Treg-driving conditions in the presence of mCRAMP. The development of Th17 cells and regulatory T lymphocytes both require TGFβ[49,385]. These results therefore suggest that mCRAMP may interact with, or boost, TGFβ-derived signals.

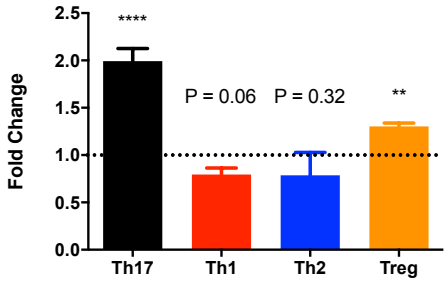


Figure 3.18: The effects of mCRAMP on the differentiation of other T helper subsets. Whole single cell splenic suspensions were cultured under Th17- (n = 27), Th1- (n = 6) or Th2-driving (n = 6) conditions, with or without 2.5 μM synthetic mCRAMP. $CD4^+$ T cells were cultured under Treg-driving conditions, with or without 2.5 μM synthetic CRAMP (n = 4). Fold change in the percentage of $CD4^+$ $IL\text{-}17A^+$ (Th17), $CD4^+$ $IFN\gamma^+$ (Th1), $CD4^+$ $IL\text{-}4^+$ (Th2) or $CD4^+$ $FOXP3^+$ (Treg) expression was determined. Data shown is mean +/- standard error. Statistical significance (where ** represents < 0.001 and **** < 0.0001) was determined using a paired t-test (UT vs. CRAMP).

3.3.13 mCRAMP requires TGFβ to increase RORγt expression and enhance Th17 differentiation in vitro

mCRAMP had no effect on the frequency of $CD4^+$ $IL\text{-}17A^+$ T lymphocytes when cultured under non-lineage-driving conditions in the absence of IL-6, IL-23 and TGFβ (**Figure 3.5**). This suggests that the peptide requires at least one, or all, of the Th17-polarizing cytokines to exert its effects. Furthermore, a small increase in Treg differentiation was observed when $CD4^+$ T cells were cultured with a high concentration of TGFβ and synthetic mCRAMP. I therefore hypothesised that mCRAMP interacts with, or boosts, signals from TGFβ.

To explore this possibility, I cultured whole single cell splenic suspensions with each cytokine alone, or by systematically omitting each one at a time. By doing so, I sought to highlight not only the importance of TGFβ in mCRAMP-induced changes, but also determine whether the addition of synthetic mCRAMP could restore a Th17-inducing environment in the absence of IL-6, IL-23 or TGFβ.

Figure 3.19 A shows that mCRAMP failed to increase the geometric mean of RORγt expression when the cells were cultured in the absence of TGFβ. This supports the hypothesis that mCRAMP requires TGFβ to enhance Th17 differentiation.

However, while mCRAMP failed to increase the percentage of $CD4^+$ $ROR\gamma t^+$ T cells on day 1 when the cells were cultured with IL-6 and IL-23 (but in the absence of TGFβ), mCRAMP significantly increased the percentage of $CD4^+$ $ROR\gamma t^+$ T cells under all other conditions, including when the cells were generated in the presence of IL-6 or IL-23 alone (**Figure 3.19 B**). One possible explanation for this is that the increase in the proportion of $CD4^+$ $ROR\gamma t^+$ T cells when cultured with only IL-6 or IL-23 (and mCRAMP) reflects an increasing number of $CD4^+$ T lymphocytes transiently expressing very low levels of RORγt, which is likely insufficient to have any downstream effects on IL-17 production.

It should be noted that an increase in the percentage of $CD4^+$ $ROR\gamma t^+$ T cells did not necessarily translate into an increase in the number of IL-17-producing $CD4^+$ T lymphocytes (**Figure 3.19 C**). RORγt and STAT3 act cooperatively with several other transcription factors (e.g. IRF4, BATF) on the *Il17* locus to promote its transcription[386]. It is therefore likely that all three Th17-polarizing cytokines are required to provide an optimal environment in which mCRAMP can promote the expression of this cytokine.

Nonetheless, taken together, these results support the hypothesis that mCRAMP requires TGFβ to boost Th17 differentiation.

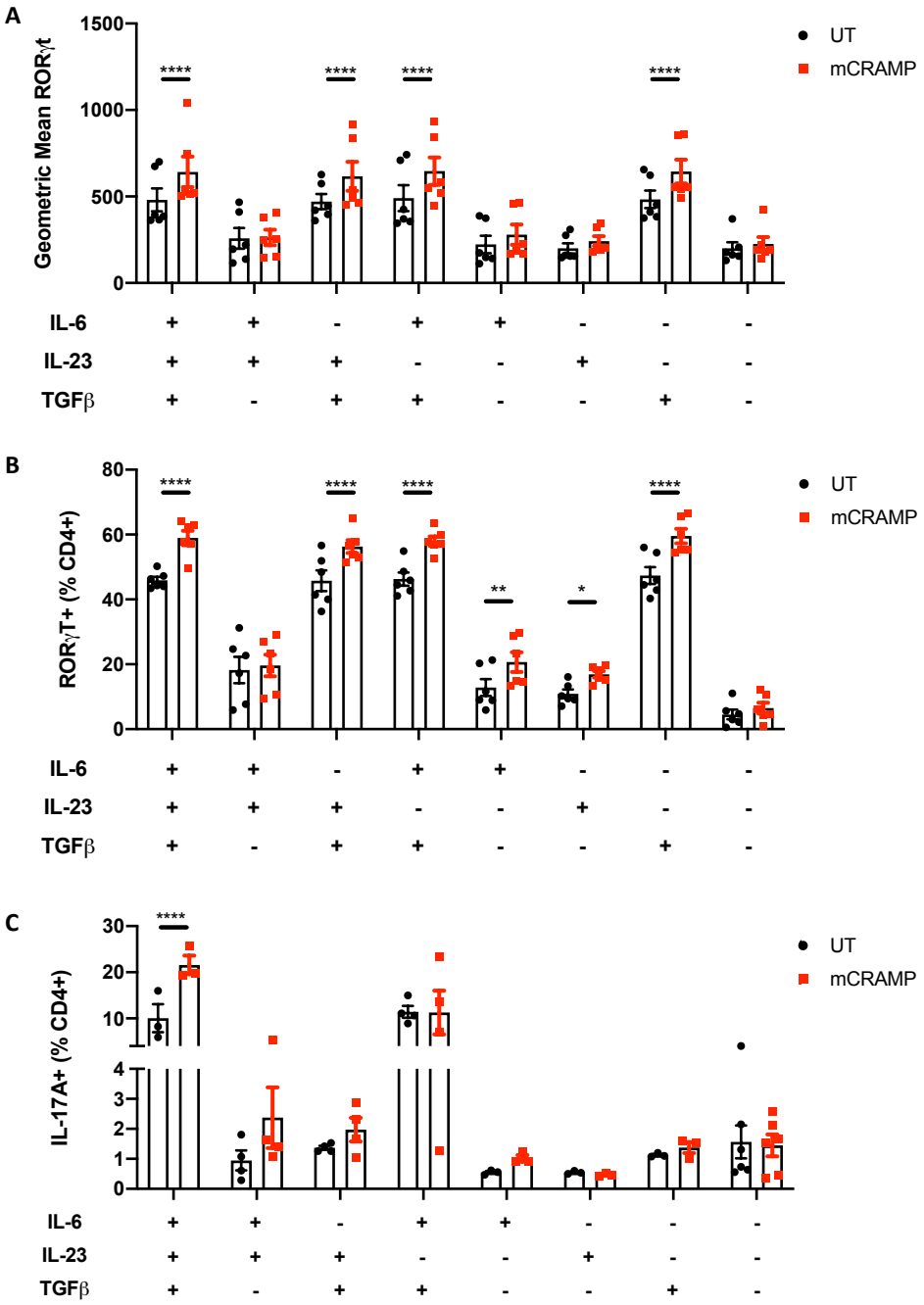


Figure 3.19: mCRAMP fails to increase RORγt expression in the absence of TGFβ. Whole single cell splenic suspensions were cultured with different combinations of IL-6 (20 ng/mL), IL-23 (20 ng/mL) and TGFβ (3 ng/mL), with or without 2.5 μM synthetic mCRAMP, for up to 2 days **(A)** Geometric mean of RORγt expression by $CD4^+$ T cells on day 1 **(B)** Percentages of $CD4^+$ $ROR\gamma t^+$ T cells on day 1 **(C)** Percentages of $CD4^+$ $IL\text{-}17A^+$ T cells on day 2. Data shown is mean +/- standard error. N = 6. Statistical significance (where * represents < 0.05, ** < 0.01 and **** < 0.0001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

3.3.14 The effects of mCRAMP on CD4$^+$ T cells are not receptor-mediated

Cathelicidin has been shown to activate a multitude of receptors to exert its immunomodulatory effects, including P2X7R, FPR2 and EGFR[387]. To determine whether mCRAMP acted via a receptor to enhance Th17 differentiation, I cultured whole single cell splenic suspensions under Th17-driving conditions, with or without synthetic mCRAMP, LL-37, the D-enantiomer of LL-37 (D-LL-37) or scrambled LL-37 (**Figure 3.20**).

mCRAMP and LL-37 show significant homology in structure and sequence identity and as a result, often display similar effects[388]. Scrambled LL-37 has the same peptide sequence as LL-37 but in an incorrect order, which can result in a loss of its helix-forming properties. D-LL-37 maintains its α-helical configuration but each amino acid is in the D-configuration (cannot be superimposed on its mirror image). I hypothesized that if the effects of mCRAMP were receptor-dependent, D-LL-37 and the scrambled peptide would not increase the frequency of CD4$^+$ IL-17A$^+$ T cells due to a loss of chirality, structure and/or sequence specificity, which would prevent binding.

As predicted, LL-37 increased the percentage of CD4$^+$ IL-17A$^+$ T cells from 9.71% (+/- 1.44) to 19.82% (+/- 0.80), similarly to mCRAMP (19.67% +/- 2.22). Surprisingly, both scrambled LL-37 and D-LL-37 also increased the generation of IL-17-producing CD4$^+$ T lymphocytes to 17.13% (+/- 1.48) and 17.65% (+/- 0.99), respectively.

These data suggest that it is highly unlikely that mCRAMP acts directly on CD4$^+$ T cells via a specific surface receptor to boost Th17 differentiation.

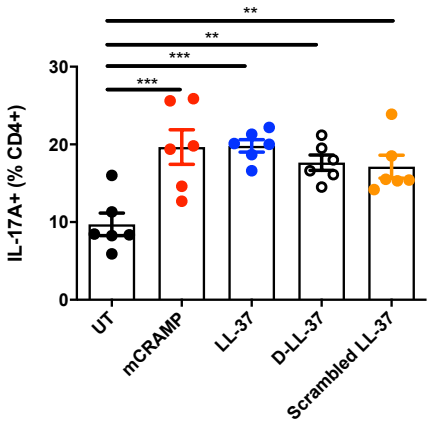


Figure 3.20: D-LL-37 and scrambled LL-37 increase Th17 differentiation. Whole single cell splenic suspensions were cultured under Th17-driving conditions, with or without 2.5 μM synthetic CRAMP, LL-37, D-LL-37 or scrambled LL-37, for 2 days**.** Percentages of $CD4^+$ $IL\text{-}17A^+$ T cells on day 2. Data shown is mean +/- standard error. N = 6. Statistical significance (where ** represents < 0.01 and *** < 0.001) was determined using an ordinary one-way ANOVA with a Dunnett's multiple comparison post-test. UT: untreated.

3.4 Discussion

Pilot data from the laboratory determined that mCRAMP-deficient mice were incapable of generating a type-17 response during inflammation induced by heat-killed *S. typhimurium* (HKST). Emily Gwyer Findlay demonstrated that T cell numbers in both primary (thymus) and secondary (spleen, inguinal lymph nodes) lymphoid organs were not significantly different between naïve WT and mCRAMP KO animals (unpublished data, Emily Gwyer Findlay). I have shown that $CD4^+$ T cells from naïve mCRAMP-deficient mice displayed relatively normal cytokine responses (IL-17A, IL-17F, IFNγ, IL-22 and GM-CSF) at resting state. Furthermore, $CD4^+$ T lymphocytes from mCRAMP KO mice were capable of producing IL-17 *in vitro* in response to exogenous cytokines and to the same extent as WT T cells. Taken together, this indicates that $CD4^+$ T lymphocytes that develop in the absence of mCRAMP do not possess an obvious underlying defect that is responsible for their inability to produce IL-17 *in vivo*. Nevertheless, the development of the Th17 response is impaired upon stimulation, such as during inflammation induced by HKST.

3.4.1 mCRAMP enhances Th17 differentiation

I sought to examine the effects of synthetic mouse cathelicidin (mCRAMP) on the differentiation of Th17 cells *in vitro*. A standard, previously published, Th17 differentiation protocol (TGFβ/IL-6) was chosen to determine the effects of synthetic mCRAMP on the development of IL-17-producing $CD4^+$ T cells[385,389,390]. IL-23 was added to cultures due to its known roles in maintaining and stabilising the Th17 phenotype[76]. Whole single cell splenic suspensions were used, as opposed to purified naïve $CD4^+$ T cells, as it was unknown whether mCRAMP acted directly or indirectly on this cell type. Phenotypic analysis was carried out ex vivo and on days 1 to 3 and it was determined that Th17 polarization was achieved by day 2 (measured by the percentage of $CD4^+$ $IL\text{-}17^+$ T cells). As such, further experiments were typically terminated on day 2, which allowed for the examination of the early effects of the peptide. However, additional studies should be carried out in the future to determine the consequences of mCRAMP exposure at later time points, particularly with regards to Th17 plasticity.

The concentration of mCRAMP used throughout this study (2.5 μM, approximately 10 μg/mL) is physiologically relevant during inflammation. For example, human cathelicidin (LL-37) is present in airway secretions from healthy newborns at around 5 μg/mL and can rise to between 10 to 30 μg/mL in infants with systemic or pulmonary infections[391]. LL-37 has also been detected in BAL fluid at 5 μg/mL in healthy individuals but this can rise to 30 μg/mL in cystic fibrosis patients[285]. However, levels of cathelicidin can increase even further in certain inflammatory conditions: Ong et al. measured a concentration of around 300 μM (approx. 1.2 mg/mL) LL-37 in psoriatic plaques[310]. In comparison, the amount of human β-defensin 2 present was only around 20 μM[310]. Nonetheless, it is important to note that a gradient of cathelicidin will surround leukocytes at a site of infection/inflammation and that the local concentration could be much higher[285].

3.4.1.1 mCRAMP increases $CD4^+$ T cell activation

mCRAMP increased the activation status of $CD4^+$ T lymphocytes cultured under Th17-driving conditions. For example, mCRAMP increased the percentage of $CD4^+$ $CD62L^-$ $CD44^+$ T

lymphocytes in Th17 cultures. Schumann et al. have demonstrated a role for CD44 in the development of Th17 cells. More specifically, they identified three distinguishable populations of $CD4^+$ T lymphocytes following stimulation with allogeneic DCs: $CD44^+$, $CD44^{++}$ and $CD44^{+++}$[370]. IL-17-producing T helper cells were mainly $CD44^{+++}$, whereas $IFN\gamma^+$ T lymphocytes were $CD44^{++}$[370]. Furthermore, blocking CD44 resulted in decreased IL-17 secretion but had no discernible effect on $IFN\gamma$[370]. Increased CD44 expression in response to mCRAMP could therefore contribute to the amplification of Th17 development.

mCRAMP also significantly increased the geometric mean of PD1. PD1 is expressed by T cells following TCR engagement and eventually declines following acutely resolved antigen encounter[392]. However, its expression is maintained in chronic disease settings, which has been associated with a progressive loss of function[392]. For example, high PD1 expression by human $CD4^+$ T lymphocytes identifies a population of exhausted effector cells that are enriched in malignant cancer[393]. Nevertheless, PD1 expression status alone cannot discriminate between exhausted and activated T cells[392]. mCRAMP increased CD44 and decreased CD62L expression, both hallmarks of an activated T cell. Moreover, two days in culture is unlikely to result in an exhausted phenotype, which is supported by the fact that $CD4^+$ T cells were still producing cytokines and proliferating normally. This data therefore indicates that upregulated PD1 expression by $CD4^+$ T lymphocytes in response to mCRAMP represents increased T cell activation and not dysfunction. However, longer term cultures (up to 14 days) could be performed to formally determine whether mCRAMP has a significant effect on T cell exhaustion.

On the other hand, it could be argued that my *in vitro* Th17 cultures simply might not contain cells expressing the PD1 ligand (PDL1). PDL1 expression is upregulated following exposure to cytokines such as $IFN\gamma$ and elevated levels are displayed on the surface of tumour cells[394]. Activation of the PD1-PDL1 pathway leads to an arrest in T cell proliferation and apoptosis[395]. In the absence of $PDL1^+$ cells, $PD1^+$ T lymphocytes might not receive the required signalling that results in T cell exhaustion.

3.4.1.2 mCRAMP increases the frequency of CD4$^+$ IL-17$^+$ T cells in Th17 cultures

mCRAMP significantly and concentration-dependently increased the percentages of CD4$^+$ RORγt$^+$ and CD4$^+$ IL-17A$^+$ T cells. RORγt expression is necessary for driving Th17 polarization and the transcription of *Il17* in response to IL-6 and TGFβ[43]. The upregulation of RORγt in Th17 cultures therefore accurately reflects changes in the frequency of IL-17 producing lymphocytes induced by mCRAMP. However, an increase in the percentage of CD4$^+$ RORγt$^+$ T cells did not necessarily translate into IL-17 production if one or two of the three Th17-stimualting cytokines were absent. RORγt and STAT3 act cooperatively with several other transcription factors (e.g. IRF4, BATF) on the *Il17* locus to promote its transcription[386]. It is therefore likely that all three Th17-polarizing cytokines are required to provide an optimal environment in which mCRAMP can promote the expression of this cytokine.

Furthermore, TGFβ alone was sufficient to induce percentages of CD4$^+$ RORγt$^+$ T lymphocytes, akin to those observed when culturing the cells with all three cytokines. However, this did not result in the development of IL-17-producing CD4$^+$ T lymphocytes. Manel et al. demonstrated that TGFβ upregulates RORγt expression in human CD4$^+$ T cells but suppresses its ability to induce IL-17 expression unless another inflammatory cytokine is present to relieve this inhibition[71]. This is in accordance with my results.

Elevated levels of IL-17F were observed in samples treated with mCRAMP. There is a significant degree of sequence homology and functional similarity between IL-17A and IL-17F, particularly in terms of their regulation and ability to induce chemokines that are important in neutrophil recruitment and activation[396]. Both genes are localised in the same chromosome region and require STAT3 and RORγt for their expression[397]. The polarization of polyclonally activated naïve CD4$^+$ T cells with TGFβ and IL-6 induces a large proportion of IL-17A and IL-17F co-expressing lymphocytes, whereas Th17 cells differentiated in the presence of IL-1β and IL-6 mainly express IL-17A[398]. In the present study, the vast majority of CD4$^+$ IL-17A$^+$ T cells were also IL-17F$^+$, which is in line with these observations made using a TGFβ/IL-6 differentiation protocol.

Interestingly, mCRAMP had no significant effect on the percentage of IL-17A single-positive lymphocytes but significantly increased the frequency of CD4$^+$ IL-17A$^-$ IL-17F$^+$ cells and IL-

17A/IL-17F dual-producers. There is only a limited amount of published data looking at the differential effects of Th17 modulators on the production of IL-17A compared to IL-17F. For example, Piccinni and colleagues demonstrated that medroxyprogesterone acetate (MPA) has no effect on IL-17F, but suppresses immune function by decreasing the production of IL-17A in an AHR-dependent manner[399]. Conversely, polycyclic aromatic hydrocarbons have been shown to increase AHR expression, which promotes IL-17A but not IL-17F production in allergic asthmatic patients[400].

According to the manufacturer's technical specifications, the antibodies I used for the intracellular detection of IL-17A and IL-17F by flow cytometry recognise both homodimers, as well as the IL-17A/F heterodimer. However, based on my staining, it was not possible to distinguish T cells that express the heterodimer from IL-17A/IL-17F co-expressing cells. Future investigations should therefore take advantage of antibodies that specifically recognise the heterodimeric form so as to evaluate the effects of mCRAMP on its production.

3.4.1.3 Are Th17 cells generated in the presence of mCRAMP more or less pathogenic?

Differentiation of Th17 cells in the presence of IL-1β (instead of TGFβ) is known to preferentially lead to the generation of pathogenic T cells that favour production of IL-17A over IL-17F[398,401]. This has led some to speculate that IL-17A$^+$ Th17 lymphocytes are more pathogenic than those that are IL-17F single- or double-positive[398,401]. Indeed, the majority of encephalitogenic CD4$^+$ T cells in the CNS during EAE express only IL-17A[398]. Moreover, IL-23 promotes the pathogenicity of Th17 cells by increasing IL-17A production[398,402]. I found that mCRAMP had no significant effect on the percentage of IL-17A single-positive lymphocytes but significantly increased the frequency of IL-17F$^+$ and IL-17A/IL-17F double-producing cells. Esplugues et al. demonstrated that the systemic application of anti-CD3 antibodies triggers the generation of "regulatory Th17" cells (rTh17) in the lamina propria, which are thought to represent the IL-17A/F double-positive cells found in the TGFβ/IL-6 differentiation protocol[398,403]. One might therefore speculate that mCRAMP promotes the development of a less pathogenic population of Th17 cells. Indeed, there was a trend suggesting that the amount of IL-17 produced by CD4$^+$ IL-17$^+$ T cells in mCRAMP-treated Th17 cultures was decreased compared to their untreated counterparts. In the future, it will be

interesting to see whether the peptide has any effect on IL-17A and IL-17F production when the cells are cultured with IL-1β and IL-6. This would help determine whether or not our observations are based purely on the protocol used for Th17 polarization.

In addition, mCRAMP had no effect on IL-22 production by $CD4^+$ T cells cultured under Th17-polarizing conditions. IL-22-secreting Th17 cells induced in response to IL-23 have been shown to specifically identify a highly pathogenic population of auto-aggressive T cells in EAE[371,402,404]. The *Il22* locus is located in proximity to *Ifng* and displays differential regulatory requirements[405]. More specifically, IL-22 expression is more dependent on AHR than RORγt and is induced by IL-6 but inhibited by high concentrations of TGFβ[79,116]. The lack of an increase in IL-22 production in response to mCRAMP demonstrates specificity of the peptide in enhancing cytokine production by Th17 cells. Furthermore, this could be explained by the fact that my results suggest that mCRAMP boosts TGFβ signals in order to enhance Th17 differentiation. It may also imply that mCRAMP does not increase the pathogenicity of developing Th17 lymphocytes, or at least not by inducing IL-22 production.

To test whether the pathogenicity of Th17 cells is altered by exposure to mCRAMP, one could perform a T cell transfer model of colitis, in which $CD4^+$ T cells cultured under Th17-driving conditions, with or without synthetic mCRAMP, are injected intraperitoneally into immunodeficient $RAG^{-/-}$ mice[406]. If Th17 lymphocytes generated in the presence of this host defence peptide are indeed less pathogenic, I would hypothesise that animals receiving mCRAMP-treated cells would display delayed onset of colitis and/or potentially lower disease scores than controls.

3.4.2 Mechanism of action

3.4.2.1 mCRAMP acts directly on $CD4^+$ T cells to enhance Th17 differentiation

Original experiments examining the effects of mCRAMP on Th17 differentiation were performed using whole single cell splenic suspensions. It was therefore unclear whether mCRAMP acted directly on the $CD4^+$ T lymphocytes, or indirectly via a different cell type such as dendritic cells (DCs). I have shown that mCRAMP had no effect on the phenotype of

differentiated DCs, as determined by the surface expression of various markers involved in T cell activation (CD86) and cross-presentation (XCR1, CLEC9A).

Nonetheless, the possibility that mCRAMP influences the differentiation and phenotype of immature DCs to further enhance Th17 differentiation cannot be excluded. A novel role for cathelicidin in directing the expansion and differentiation of DCs in culture towards an enhanced $CD141^+/CD103^+$-like phenotype was recently identified[338]. Several studies have shown that $CD103^+$ DCs drive Th17-mediated immunopathology[377,407]. For example, Zelante et al. found that $CD103^+$ DCs in the lung produce IL-2, which promotes the development of an optimally protective Th17 response during invasive pulmonary aspergillosis[407]. Moreover, $CD103^+$ DCs induce pathogenic Th17 differentiation that drives spontaneous colitis in mucin2-deficient mice[377]. However, Gwyer Findlay and colleagues demonstrated that human cathelicidin (LL-37) only enhanced $CD103^+$ DC generation when the cells were exposed within the first 24 hours of culture, indicating that the peptide modulates DC differentiation and does not simply upregulate CD103 expression on fully differentiated cells[338]. This is similar to prostaglandin E2, which alters the IL-12/IL-23 balance in DC precursors during differentiation to promote Th17 differentiation at the expense of IFNγ-secreting Th1 effector cell development[408].

Another important cell type resident in the spleen and which has been shown to direct and regulate the inflammatory response through Th17 induction is B cells. Wang and Rothstein found that activated splenic B-2 cells upregulated the expression of CD44, CD80 and CD86, which played a crucial role in promoting Th17 polarization[409]. Furthermore, IFNβ-driven inflammatory B cells produce increased levels of IL-6, which drives the differentiation of myelin-specific, pro-inflammatory Th17 cells[410]. I have shown that mCRAMP increases the activation state of T cells (CD44) and others have demonstrated how this peptide upregulates the expression of co-stimulatory molecules on other cell types such as DCs[336]. One could therefore speculate that mCRAMP may exert similar effects on B cells, which could in turn contribute to enhancing the development of IL-17-producing $CD4^+$ T lymphocytes. This could be examined in the future by examining the effects of mCRAMP on Th17 differentiation in B - T cell co-cultures.

However, mCRAMP significantly increased Th17 differentiation of sorted $CD4^+$ T cells, indicating that this host defence peptide promotes the development of IL-17-producing T lymphocytes, at least in part, by acting directly on this cell type. Nonetheless, it is possible that mCRAMP exerts additional indirect effects that I have not identified in the present study, which also contribute to this.

3.4.2.2 mCRAMP has no effect on IL-23R and IL-6R expression

Naïve $CD4^+$ T cells do not express IL-23R, which is induced by IL-6 and IL-21[75]. Conversely, IL-6R is downregulated during T cell activation[411]. However, differentiating T lymphocytes retain their responsiveness to this cytokine via IL-6 *trans* signalling, which has been implicated in the local maintenance of Th17 cells[411]. Increased expression of these receptors has been shown to promote IL-17 secretion by this T helper cell subset. For instance, Zhang et al. found that elevated IL-6R expression on $CD4^+$ T cells contributes to Th17 responses in patients with chronic hepatitis B infection[412]. Furthermore, oestrogen receptor α signalling upregulates IL-23R in Th17 cells to increase IL-17 production[413].

I found that IL-23R and IL-6R expression were not significantly different between untreated and mCRAMP-treated samples. This suggests that mCRAMP does not enhance Th17 differentiation by increasing the sensitivity of $CD4^+$ T lymphocytes to IL-6 and IL-23 by upregulating their receptors. However, the quality of staining was highly variable and the expression of TGFβR was not investigated due to the lack of a suitable flow cytometry antibody. To confirm these results, the expression of IL-23R, IL-6R and TGFβR in response to mCRAMP should therefore be examined by PCR.

3.4.2.3 mCRAMP is a $CD4^+$ T cell survival factor

Cathelicidin has been shown to suppress apoptosis of several cell types, including cardiomyocytes, endothelial cells and keratinocytes[358,414,415]. Conversely, there is only a limited amount of published data suggesting that this host defence peptide acts as a survival factor to protect cells of the immune system from death. For example, Nagaoka et al. found that human cathelicidin (LL-37), human β-defensin 3 (hBD3) and HNP-1 suppress neutrophil

apoptosis via the activation of FPR2 and P2X7R[416]. However, findings published by Li and colleagues suggested that this was probably an artefact due to rapid secondary necrosis induced by LL-37, which selectively permeabilizes apoptotic leukocytes[199,417].

Mader and colleagues demonstrated that LL-37 induces granzyme-mediated apoptosis of regulatory and cytotoxic $CD8^+$ T cells but had no effect on $CD4^+$ T lymphocytes[360,378]. This is contrary to my findings: mCRAMP significantly increased the percentage of viable $CD4^+$ T lymphocytes and decreased the percentage of necrotic cells, as determined by annexin/PI staining. One possible explanation for this divergence could be that the concentration of LL-37 used by Mader et al. was very high (40 μg/mL) and therefore potentially cytotoxic[360,378]. Nevertheless, to my knowledge, my data is the first demonstration of a neutrophil peptide increasing the survival of T cells.

Interestingly, mCRAMP protected both $CD4^+$ $IL\text{-}17^+$ and $IL\text{-}17^-$ cells from death. Furthermore, this was not a result of increased T cell proliferation, indicating that mCRAMP does not enhance Th17 differentiation by increasing the proliferative capacity of $CD4^+$ $IL\text{-}17^+$ T lymphocytes and/or by promoting the death of non-Th17 cell types.

Increasing the longevity of $CD4^+$ $IL\text{-}17^+$ T cells could result in a prolonged period where these cells are able to produce inflammatory cytokines *in vivo*, such as IL-17 and IL-21. IL-21 is particularly important in creating a positive feedback loop to expand Th17 cells during the self-amplification stage of differentiation[418]. I found that the increase in IL-17-producing $CD4^+$ T lymphocytes in response to mCRAMP was associated with an increase in IL-21 production. By protecting IL-21-producing $CD4^+$ $IL\text{-}17^+$ T cells from death, mCRAMP may further amplify Th17 polarization. This could, however, subsequently lead to greater immunopathology if left unchecked.

3.4.2.4 mCRAMP requires TGFβ to enhance Th17 differentiation

Under non-lineage driving conditions and in the absence of Th17-polarizing cytokines, mCRAMP did not increase the percentage of $CD4^+$ $IL\text{-}17A^+$ T cells. One possible explanation for this is that the peptide exerts its effects by interacting with or boosting signals from IL-6,

IL-23 and/or TGFβ. However, mCRAMP did transiently increase the geometric mean of PD1 and the percentage of $CD4^+$ $CD62L^-$ $CD44^+$ T cells on day 1. This indicates that there are at least two, separate T cell signalling pathways induced by mCRAMP, one of which is switched on in all conditions and one which is Th17-specific.

mCRAMP did not have a significant effect on the differentiation of other T helper subsets, namely Th1 and Th2. Conversely, a small increase in the percentage of $CD4^+$ $FOXP3^+$ T cells was observed following culture under Treg-driving conditions. Th17 cells and regulatory T lymphocytes are closely related: the differentiation of both subsets requires TGFβ[49]. More specifically, TGFβ alone and at high concentrations induces FOXP3 expression, whereas low concentrations of TGFβ, together with IL-6, enhance STAT3 activation and upregulate RORγt during the initial stages of Th17 polarization[66]. I consequently hypothesised that mCRAMP enhances the downstream effects of TGFβ signalling. For example, TGFβ promotes Th17 development by suppressing SOCS3, a negative regulator of STAT3 activation, as well as T-bet and GATA3, thereby inhibiting $CD4^+$ T cells from adopting alternate Th1 and Th2 fates[65,66]. Indeed, when whole single cell splenic suspensions were cultured in the absence of TGFβ, mCRAMP had no significant effect on RORγt expression.

Several studies have shown that Th17 cells display late developmental plasticity, which allows for functional adaptation to different physiological situations during an immune response[419]. For example, using lineage-tracing mice, Gagliani and colleagues demonstrated that Th17 cells acquire the transcriptional profile and potent suppressive functions of Tr1 cells (produce IL-10 but are $FOXP3^-$) during the resolution phase of Th17-mediated colitis[420]. Sustained TGFβ signalling is essential for maintaining the expression of IL-17A and IL-17F: re-stimulation of committed Th17 precursors with IL-12 and in the absence of TGFβ promotes the emergence of IFNγ-producing cells that lack IL-17 expression[421]. Another potential mechanism through which mCRAMP could promote Th17 differentiation is therefore by preventing the transdifferentiation of this T helper cell subset by maintaining TGFβ-derived signals.

Wanke et al. demonstrated that TGFβ drives the development of $IL\text{-}17F^+$ and $IL\text{-}17A^+$ $IL\text{-}17F^+$ T cells[398]. Furthermore, they suggested that IL-17A and IL-17F double-producers represent a

regulatory subset of Th17 cells known to be induced in response to TGFβ[398,403]. As discussed previously, I have shown that mCRAMP displayed a more pronounced effect on IL-17F expression than IL-17A. Taken together, this provides further evidence to support the hypothesis that mCRAMP enhances TGFβ signals to promote the generation of a less pathogenic population of Th17 lymphocytes.

There is a limited amount of previously published data linking host defence peptides to TGFβ. For example, the TGFβ homologue Dpp, found in *Drosophila melanogaster*, is increased in the gut following bacterial entry and contributes to the developing immune response and control of bacterial homeostasis by enhancing antimicrobial peptide production[422]. On the other hand, Scott et al. found that human cathelicidin (LL-37) increases the expression of TGFβR1 by macrophages[353]. Furthermore, mCRAMP has been shown to decrease TGFβ-induced collagen synthesis in keloid fibroblasts and inhibit TGFβ signalling in diabetic hearts[423].

3.4.2.5 The effects of mCRAMP on Th17 differentiation are not receptor-mediated

Cathelicidin activates a variety of different receptors depending on cell type and context, including P2X7R, FPR2 and EGFR[387]. However, I have shown that both the D-enantiomer and scrambled cathelicidin peptide increased the percentage of $CD4^+$ $IL\text{-}17A^+$ T cells to the same extent as mCRAMP, suggesting that a structurally unique ligand binding site is not involved.

One possible explanation for this is that mCRAMP inserts itself into the membrane and interacts with the cytoplasmic C' terminal end of a receptor, similarly to its activation of P2X7R[387]. D-LL-37, in which each amino acid is in the D-configuration, LL-37 and mCRAMP possess the same α-helical configuration; the hydrophobic environment of the membrane allows for specific interactions to be formed between polypeptides, irrespective of their chirality[424]. Interestingly, modifications of the physical properties of the lipid membrane bilayer resulting in decreased membrane fluidity have been proposed as a potential mechanism for the activation of the calcium-permeable channel TRPV2 in breast cancer cells by LL-37[425]. Furthermore, both the D- and L-enantiomer of LL-37 displayed identical effects in this study[425]. However, scrambled LL-37, in which the same amino acids are ordered

randomly, maintains its net positive charge but loses its propensity to fold into the correct amphipathic structure. This would theoretically prevent the peptide from behaving in the same way as mCRAMP, LL-37 and D-LL-37.

Several studies (including unpublished observations from other group members) have reported limited activity of the scrambled peptide with regards to its antimicrobial function[426]. For instance, Gordon et al. found that scrambled LL-37 demonstrated limited inhibition of herpes simplex virus type 1 (HSV-1) relative to a PBS control, but significantly less than that of LL-37[426]. They proposed that this is due to the fact that the action of cathelicidin is dependent not only on size and sequence, but also charge, degree of helicity, overall hydrophobicity, amphipathicity and the angles subtended by hydrophobic and hydrophilic surfaces[426]. It is therefore possible that the effects of mCRAMP on Th17 differentiation are not dependent on its helical structure but are instead mediated by something like charge. This would be in contrast to previously published reports, in which it was shown that the cationicity of a peptide is not sufficient to induce bacterial membrane permeabilization and killing[417]. Nevertheless, this could be tested in the future by examining the effects of other short positive peptides of random sequence and structure.

Cathelicidin can also be taken up by cells in a non-specific manner in which a receptor is not required[333,334]. For instance, LL-37 can bind to extracellular DNA plasmids through electrostatic interactions and target them to the nuclear compartment of mammalian cells (human embryonic fibroblasts, bladder carcinoma cells)[333]. A similar phenomenon involving lipid rafts and proteoglycan-dependent endocytosis could therefore be required for the uptake of mCRAMP by $CD4^+$ T lymphocytes.

Finally, unpublished data from the laboratory has shown that human $CD4^+$ T cells incubated with TAMRA-labelled LL-37 rapidly take up the peptide, which locates to the nucleus typically within 10 minutes (unpublished observation by Emily Gwyer Findlay & Brian McHugh). Several other groups have shown that cathelicidin can localize to the nucleus in other cell types. For example, Lau and colleagues demonstrated that LL-37 is actively taken up by A549 epithelial cells and eventually localises to the perinuclear region[342]. Like most AMPs, cathelicidins are highly cationic and as a result, are capable of binding to negatively-charged

DNA[285]. For example, LL-37 binds to inert self-DNA in psoriatic lesions to form aggregated and condensed structures that are delivered to early endocytic compartments in pDCs[334]. Muñoz et al. demonstrated that following migration to the nucleus, LL-37 can bind to gene promoter regions and directly modulate the transcriptional program of A375 melanoma cells[427]. mCRAMP may therefore enhance Th17 differentiation by acting as a transcription factor to promote the development of this T helper cell subset. However, it is highly unlikely that scrambled LL-37 would be able to do this, which suggests that this is not how these peptides boost the development of IL-17-producing $CD4^+$ T lymphocytes.

3.5 Summary

To summarize, I have shown that T cells that develop in the absence of mouse cathelicidin (mCRAMP) do not possess an obvious underlying defect that is responsible for their inability to produce IL-17 *in vivo* in response to inflammation induced by heat-killed *S. typhimurium*.

Furthermore, mCRAMP is a Th17 differentiation enhancing factor, which acts directly on T cells *in vitro* to increase their activation status, viability and production of IL-17. Whilst I suggest that there are at least two, separate T cell signalling pathways induced by the peptide (one of which is non-specific and one which is Th17-specific), I have provided evidence that indicates mCRAMP requires TGFβ to promote the development of a less pathogenic population of Th17 lymphocytes. Finally, I predict that these effects are not receptor-mediated due to the fact that both the D-enantiomer and scrambled peptide display similar effects compared to mCRAMP.

CHAPTER 4

mCRAMP skews T helper cell differentiation away from the Th1 lineage and enhances Th17 polarization via the aryl hydrocarbon receptor

4.1 Introduction

I have shown that mCRAMP enhances mouse Th17 differentiation *in vitro* by acting directly on $CD4^+$ T cells to increase RORγt expression and promote the development of IL-17-producing lymphocytes. However, the exact mechanism through which this occurs remains unclear. For instance, Lau and colleagues demonstrated that human cathelicidin (LL-37) is actively taken up by A549 lung epithelial cells and eventually localises to the perinuclear region[342]. Furthermore, LL-37 is rapidly endocytosed and localizes to the nucleus of $CD4^+$ T lymphocytes (unpublished observation by Emily Gwyer Findlay & Brian McHugh). Based on these observations, it has been suggested that cathelicidin can directly modulate gene expression by acting as a transcription factor[427].

On the other hand, it is also possible that mCRAMP may amplify type-17 responses by inducing metabolic reprogramming. For example, glycolysis is especially important for the development of Th17 lymphocytes: defective glycolysis can drastically impair the proliferation and cytokine production of this T helper cell subset[428,429]. The transcription factor, HIF1α (hypoxia-inducible factor 1α), plays a crucial role in mediating glycolytic activity and is selectively expressed in Th17 lymphocytes, in which it regulates the expression of Th17 signature genes through direct transcriptional activation of RORγt[429,430]. HIF1α induction requires signalling through mTOR (mammalian target of rapamycin), another central regulator of cellular metabolism[429]. Impaired mTOR signalling in naïve $CD4^+$ T cells results in their failure to differentiate into Th17 lymphocytes[431]. Interestingly, cathelicidin has been shown to enhance the LPS-induced phosphorylation of mTOR in a keratinocyte cell line[432]. Furthermore, GAPDH is another enzyme that plays a crucial role in glycolysis and has also been identified as a novel intracellular receptor for LL-37 in macrophages[428,433].

I have shown that mCRAMP requires TGFβ to amplify Th17 differentiation *in vitro*. TGFβ induces multiple pathways that promote the development of Th17 cells, many of which could be enhanced by mCRAMP[434]. For instance, TGFβ receptor signalling phosphorylates SMAD2 and SMAD3, which form a heterotrimeric complex with SMAD4 that translocates to the nucleus[434]. SMAD4 interacts with the SKI repressor to suppress Th17 differentiation through direct binding to *Rorc*[434,435]. This is offset by TGFβ, which triggers degradation of SKI[435]. Other,

SMAD-independent pathways activated by TGFβ that promote Th17 differentiation include the suppression of SOCS3, the activation of RhoA/ROCK2 and the inhibition of Eomes[434]. SOCS3 is a negative regulator of STAT3: small interfering RNA knockdown of SOCS3 promotes *Rorc* expression and *in vitro* Th17 differentiation[66]. ROCK2 is required for the phosphorylation of IRF4, which promotes the expression Th17-related genes including *Rorc* and *Il17*[434,436]. TGFβ also suppresses Eomes through the Jun N-terminal kinase-c-Jun pathway[437]. Eomes directly inhibits RORγt and IL-17A: ablation of Eomes expression by short hairpin RNA induces Th17 differentiation, whereas overexpression of this transcription factor constrains Th17 polarisation[437].

4.2 Aims

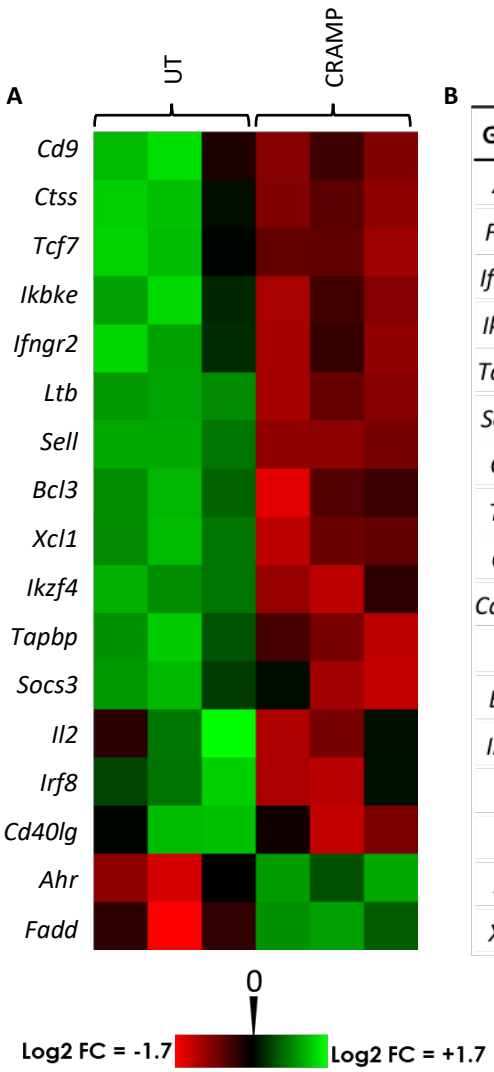
mCRAMP has the potential to act through a wide variety of mechanisms to promote Th17 differentiation. I hypothesised that exposure to mCRAMP alters the transcriptional program of $CD4^+$ T cells to enhance the development of IL-17-producing T lymphocytes. I therefore analysed the gene expression profile of $CD4^+$ T cells cultured under Th17-driving conditions for 24 hours, with or without synthetic mCRAMP, to help dissect the mechanism through which this host defence peptide acts on these cells.

4.3 Results

4.3.1 mCRAMP induces genetic changes in $CD4^+$ T cells cultured under Th17-polarizing conditions

I have shown that mCRAMP is a Th17 differentiation enhancing factor that amplifies the development of IL-17-producing $CD4^+$ T lymphocytes. I therefore sought to examine the genetic changes induced by mCRAMP that could contribute to this. To do so, I cultured whole single cell splenic suspensions under Th17-driving conditions, as previously described, with or without synthetic mCRAMP. After 24 hours of culture, I sorted $CD4^+$ T cells ($DAPI^-$ $CD4^+$ $CD8^-$; purity > 97%) and compared their gene expression profiles by Nanostring.

The results revealed that 17 genes were significantly differentially expressed and had a log2 fold change of 1 or more (i.e. 2-fold or more different). More specifically, mCRAMP significantly downregulated the expression of 15 genes and upregulated 2 genes (**Figure 4.1**; see appendix (Table A1) for the full list of differentially expressed genes).



B

Gene	Protein	Log2 FC	FC	P-value
Ahr	AHR	1.72	3.29	0.016
Fadd	FADD	1.01	2.01	0.047
Ifngr2	IFNɣR2	-1.01	-2.01	0.008
Ikbke	IKBKE	-1.02	-2.03	0.009
Tapbp	Tapasin	-1.03	-2.04	0.003
Socs3	SOCS3	-1.04	-2.06	0.026
Ctss	Cathepsin S	-1.08	-2.11	0.005
Tcf7	TCF1	-1.12	-2.17	0.007
Cd9	CD9	-1.13	-2.19	0.015
Cd40lg	CD40 ligand	-1.21	-2.31	0.029
Irf8	IRF8	-1.44	-2.71	0.033
Bcl3	BCL3	-1.46	-2.75	0.014
Ikzf4	EOS	-1.49	-2.80	0.002
Il2	IL-2	-1.7	-3.25	0.066
Ltb	Lymphotoxin B	-1.71	-3.27	4.82E-05
Sell	CD62L	-1.76	-3.39	6.05E-05
Xcl1	XCL1	-1.95	-3.86	0.0004

Figure 4.1: mCRAMP induces genetic changes in CD4⁺ T cells cultured under Th17 driving conditions. Nanostring analysis was performed in CD4⁺ T cells that had been cultured for 24 hours under Th17 driving conditions, with or without 2.5 μM synthetic mCRAMP **(A)** Heat map of significantly changed genes in untreated and CRAMP-treated samples **(B)** Table presenting statistically significantly differentially expressed genes in response to mCRAMP. N = 3. UT: untreated; FC: fold change.

4.3.1.1 mCRAMP modulates the expression of genes associated with T cell activation

The Nanostring analysis revealed that *Sell*, which encodes CD62L, was significantly downregulated by mCRAMP (log2 FC = -1.76). The downregulation of CD62L *in vivo* redirects lymphocytes away from the lymph nodes following antigen encounter and towards sites of infection/inflammation[369]. It is therefore commonly used to identify activated T cells, together with CD44. I found that mCRAMP increased the activation status of $CD4^+$ T cells cultured under Th17-driving conditions, which was marked by a significant increase in the percentage of $CD4^+$ $CD62L^-$ $CD44^+$ T lymphocytes (**Chapter 3: Figure 3.7**).

To validate the Nanostring data, I analysed the percentages of $CD4^+$ CD62L single-positive cells in Th17 cultures, following exposure to mCRAMP, by flow cytometry. Figure 4.2 shows that mCRAMP significantly decreased the frequency of $CD4^+$ $CD62L^+$ T lymphocytes on days 1 to 3. For example, this dropped from 36.43% (+/- 1.80) to 27.12% (+/- 1.68) on day 2. However, the geometric mean of CD62L of $CD4^+$ $CD62L^+$ T lymphocytes was no different between untreated and CRAMP-treated samples (**Figure 4.2 C**).

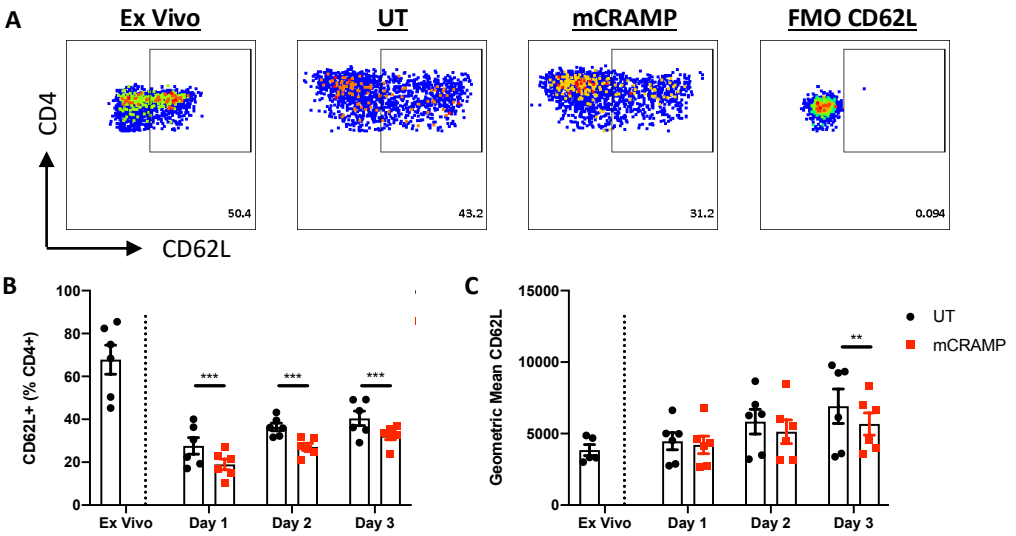


Figure 4.2: mCRAMP decreases the frequency of $CD4^+$ $CD62L^+$ T cells cultured under Th17-driving conditions. Whole single cell splenic suspensions were cultured under Th17-driving conditions, with or without 2.5 μM synthetic mCRAMP **(A)** Representative plots of CD62L expression by $CD4^+$ T cells on day 2, assessed by flow cytometry **(B)** Percentages of $CD4^+$ $CD62L^+$ T cells, *ex vivo* and days 1-3 **(C)** Geometric mean of $CD4^+$ $CD62L^+$ T cells, *ex vivo* and days 1-3. Data shown is mean +/- standard error. N = 6. Statistical significance (where * represents < 0.05 and ** < 0.01) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

4.3.1.2 mCRAMP downregulates genes involved in the negative regulation of Th17 differentiation

The expression of *Socs3* (suppressor of cytokine signalling 3) and *Tcf7* (encodes TCF1), which have both previously been implicated in the negative regulation of Th17 differentiation, were also significantly reduced in response to mCRAMP (log2 FC = -1.04 and -1.12 respectively). SOCS3 inhibits STAT3 activation and TCF1 negatively regulates the expression of the IL-17 family of cytokines[438,439]. Their downregulation suggests that mCRAMP enhances Th17 differentiation by relieving the inhibition of these molecules on the expression of genes that drive the development of IL-17-producing $CD4^+$ T cells.

4.3.1.3 mCRAMP downregulates Th1-related genes

Several classical Th1-related genes were downregulated following exposure to mCRAMP, including *Bcl3* (B-cell lymphoma 3-encoded protein; log2 FC = -1.46), *Irf8* (Interferon regulatory factor 8; log2 FC = -1.44), *Ikzf4* (IKAROS family zinc finger 4, otherwise known as EOS; log2 FC = -1.49), *Il2* (interleukin-2; log2 FC = -1.7) and *Xcl1* (X-C motif chemokine ligand 1, otherwise known as lymphotactin; log2 FC = -1.95). This suggests that mCRAMP potentially skews T helper cell differentiation away from the Th1 lineage.

Many of these Th1-related genes have been shown to exert a suppressive function on the differentiation of Th17 cells. For example, IRF8 is highly expressed in Th1 lymphocytes but also directs a silencing program for Th17 differentiation[440]. More specifically, IRF8 acts as a transcriptional inhibitor that suppresses Th17 polarization, in part, by physically interacting with RORγt[440]. I therefore sought to validate the Nanostring data by examining the expression of IRF8 in $CD4^+$ T cells by intracellular flow cytometry (**Figure 4.3**). Whole single cell splenic suspensions were cultured under Th17-driving conditions, as previously described, with or without synthetic mCRAMP.

The percentage of $CD4^+$ $IRF8^+$ T cells was significantly decreased in response to mCRAMP on days 2 and 3 (**Figure 4.3 B**). For example, this dropped from 73.17% (+/- 5.63) to 58.50% (+/- 5.18) on day 2. Furthermore, the geometric mean of IRF8 of $CD4^+$ $IRF8^+$ T lymphocytes was

also significantly reduced from 4129 (+/- 54) to 3141 (+/- 84) on day 2, as well as on day 3 (**Figure 4.3 C**).

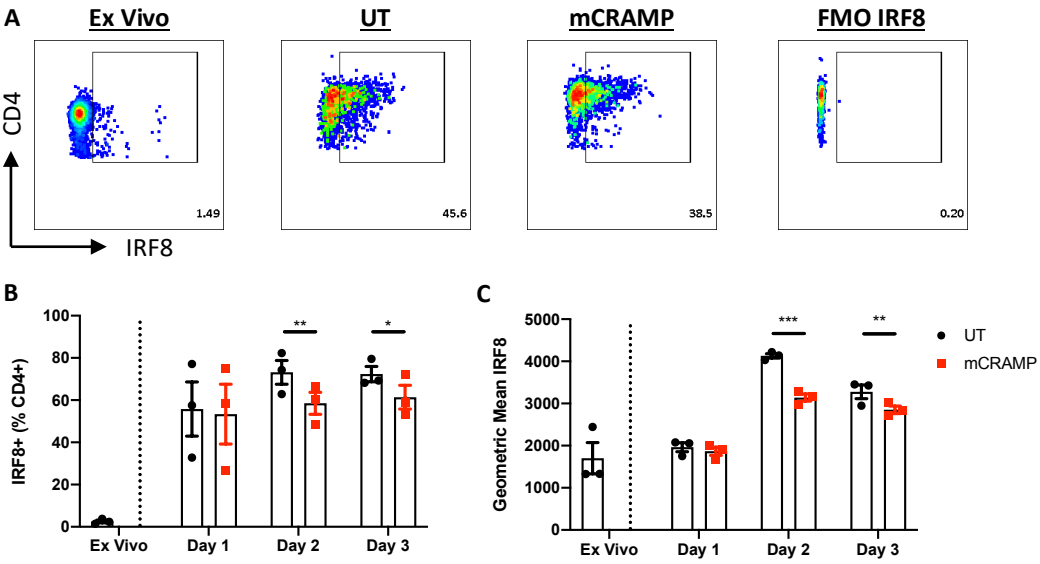


Figure 4.3: mCRAMP decreases IRF8 expression by CD4⁺ T cells. Whole single cell splenic suspensions were cultured under Th17-driving conditions, with or without 2.5 μM synthetic mCRAMP, for up to 3 days **(A)** Representative plots of IRF8 expression by CD4⁺ T cells on day 2, assessed by flow cytometry **(B)** Percentages of CD4⁺ IRF8⁺ T cells, *ex vivo* and days 1-3 **(C)** Geometric mean of CD4⁺ IRF8⁺ T cells, *ex vivo* and days 1-3. Data shown is mean +/- standard error. N = 3. Statistical significance (where * represents < 0.05, ** < 0.01 and *** < 0.001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

Another classical Th1-associated gene identified in the Nanostring analysis that has also been shown to restrain Th17 differentiation is *Il2*. IL-2 is predominantly produced by naïve T helper cells and is required for the proliferation and differentiation of precursors into effector lymphocytes[441]. This cytokine primes and maintains Th1 differentiation whilst simultaneously antagonizing Th17 development via STAT5[442].

To validate this Nanostring result, I measured the concentration of IL-2 in cell culture supernatants by ELISA (**Figure 4.4**). I observed an almost complete suppression of production when the cells were cultured under Th17-driving conditions and in the presence of mCRAMP:

the concentration of IL-2 fell from 44.36 to 18.12 ng/mL on day 2 and from 64.46 to 5.61 ng/mL on day 3.

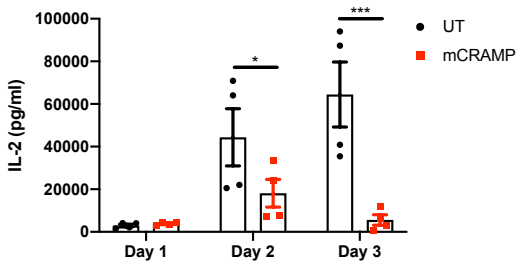


Figure 4.4: mCRAMP suppresses IL-2 production by CD4$^+$ T cells cultured under Th17-driving conditions. CD4$^+$ T cells were cultured under Th17-driving conditions, with or without 2.5 μM synthetic mCRAMP, for up to 3 days. The concentration of IL-2 in cell culture supernatants was determined by ELISA every 24 hours. Data shown is mean +/- standard error. N = 4. Statistical significance (where * represents < 0.05 and *** < 0.001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

The downregulation of genes such as IRF8 and IL-2 suggests that mCRAMP skews T helper cell differentiation away from the Th1 lineage and in doing so, also suppresses the negative regulatory networks that normally limit Th17 responses.

4.3.2 mCRAMP decreases T-bet expression and IFNγ production by CD4$^+$ T cells cultured under Th17-driving conditions

The Nanostring analysis revealed that several Th1-related genes were significantly downregulated by mCRAMP, suggesting that the peptide skews T helper cell differentiation away from the Th1 lineage. Th1 lymphocytes are the primary producers of IFNγ and their differentiation is driven, in part, by the master transcriptional regulator T-bet[26]. I therefore investigated the expression of T-bet and IFNγ by CD4$^+$ T cells in Th17 cultures, treated with or without synthetic mCRAMP (**Figure 4.5**).

Figure 4.5 shows that mCRAMP significantly decreased the frequency of T-bet-expressing CD4$^+$ lymphocytes on days 2 and 3 (**Figure 4.5 C**). For example, the percentage of CD4$^+$ RORγt$^-$ T-bet$^+$ T cells dropped from 27.04% (+/- 8.27) to 13.62% (+/- 5.03) on day 2.

Th17 cells can co-express T-bet and RORγt[381]. It has been suggested that secondary expression of T-bet is required to stabilize pathogenic IL-17 and IFNγ double-producing Th17 lymphocytes[443,444]. I also observed a significant decrease in the frequency of $CD4^+$ $ROR\gamma t^+$ T-bet^+ T cells on day 3 following exposure to mCRAMP, from 28.06% (+/- 6.50) to 18.84% (+/- 4.03) (**Figure 4.5 D**).

The reduction in T-bet expression by $CD4^+$ T lymphocytes cultured in the presence of mCRAMP was associated with a significant decrease in IFNγ-producing cells: the percentage of $CD4^+$ $IFN\gamma^+$ T cells fell from 3.77% (+/- 0.47) to 2.06% (+/- 0.33) on day 2 (**Figure 4.5 E**), although no changes in the geometric mean of IFNɣ were detected (**Figure 4.5 F**). Furthermore, no IL-17/IFNγ double-producing lymphocytes were identified.

Interestingly, mCRAMP did not decrease the percentage of dead $CD4^+$ $IFN\gamma^+$ T lymphocytes, as determined by uptake of a fixable viability dye (**Figure 4.5 G**). This was in contrast to total $CD4^+$ and $CD4^+$ $IL\text{-}17^+$ T cells (**Chapter 3: Figure 3.16**) and suggests that mCRAMP does not protect this T helper subset from death. Furthermore, no significant differences were observed in the proliferation of $CD4^+$ $T\text{-}bet^+$ T lymphocytes between untreated and CRAMP-treated samples, as determined by CFSE dye dilution (**Figure 4.5 H**).

Taken together, these results support the hypothesis that mCRAMP skews T helper cell polarisation away from a Th1 phenotype and towards the Th17 lineage.

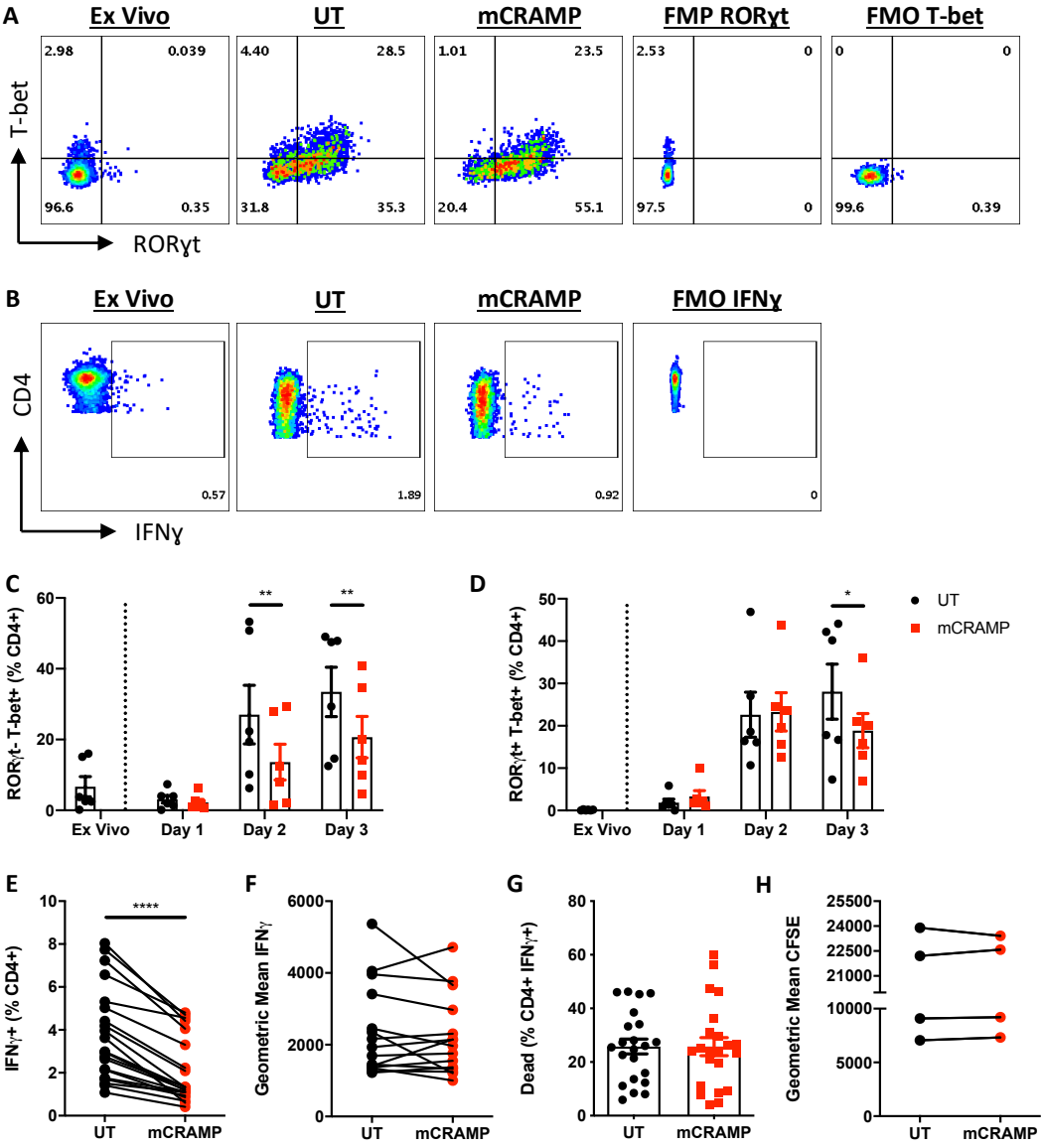


Figure 4.5: mCRAMP decreases T-bet expression and IFNɣ production by CD4⁺ T cells under Th17-driving conditions. Whole single cell splenic suspensions were cultured under Th17-driving conditions, with or without 2.5 μM synthetic mCRAMP **(A)** Representative plots of RORγt and T-bet expression by CD4⁺ T cells on day 2, assessed by flow cytometry **(B)** Representative plots of IFNɣ production by CD4⁺ T cells on day 2, assessed by flow cytometry **(C)** Percentages of CD4⁺ RORγt⁻ T-bet⁺ T cells, *ex vivo* and days 1-3 (n = 6) **(D)** Percentages of CD4⁺ RORγt⁺ T-bet⁺ T cells, *ex vivo* and days 1-3 (n = 6) **(E)** Percentages of CD4⁺ IFNɣ⁺ T cells on day 2 (n = 21) **(F)** Geometric mean of IFNɣ of CD4⁺ IFNɣ⁺ T cells on day 2 (n = 15) **(G)** Percentages of dead CD4⁺ IFNɣ⁺ T cells on day 2 (n = 22) **(H)** Geometric mean of CD4⁺ T-bet⁺ CFSE⁺ T cells on day 2. Data shown is mean +/- standard error. Statistical significance (where * represents < 0.05, ** < 0.01 and **** < 0.0001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test (C & D) or a paired t-test (E). UT: untreated.

4.3.3 mCRAMP upregulates the expression of the aryl hydrocarbon receptor

Ahr was one of only two genes identified in the Nanostring data that was significantly upregulated by mCRAMP (log2 FC = 1.72). The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that integrates environmental, dietary, microbial and metabolic cues to control transcriptional programmes in a ligand-specific, cell-type-specific and context-specific manner[445]. Moreover, AHR has been shown to play an important role in the differentiation of Th17 lymphocytes[446].

I therefore confirmed the Nanostring results by intracellular flow cytometry (**Figure 4.6**). Whole single cell splenic suspensions were cultured under Th17-driving conditions, as previously described, with or without synthetic mCRAMP. Figure 4.6 shows that mCRAMP significantly increased the percentage of $CD4^+$ AHR^+ T cells, from 6.04% (+/- 1.05) to 10.29% (+/- 1.16) on day 1 (**Figure 4.6 B**) but had no effect on the geometric mean of AHR (gated $CD4^+$ AHR^+ T lymphocytes) (**Figure 4.6 C**). This was cumulative and even more pronounced on day 2 (**Figure 4.6 D:** 75.50% +/- 1.43 compared to 54.83% +/- 1.53). However, statistical analysis revealed that the increase in $CD4^+$ AHR^+ T lymphocytes in response to the peptide was not concentration-dependent (**Figure 4.6 E**).

I have suggested that mCRAMP requires TGFβ to promote Th17 differentiation (**Chapter 3: Figure 3.19**). To investigate the importance of AHR within this pathway, I analysed AHR expression by $CD4^+$ T cells following culture in the absence of IL-6, IL-23 or TGFβ or with a single Th17-polarizing cytokine, with or without synthetic mCRAMP (**Figure 4.6 F**). Unlike the observations made previously, where in the absence of TGFβ, mCRAMP failed to increase RORγt expression, mCRAMP did not increase the percentage of $CD4^+$ AHR^+ T cells in any condition, except when all three cytokines were included. This indicates that IL-6, IL-23 and TGFβ are all required in order for mCRAMP to increase AHR expression.

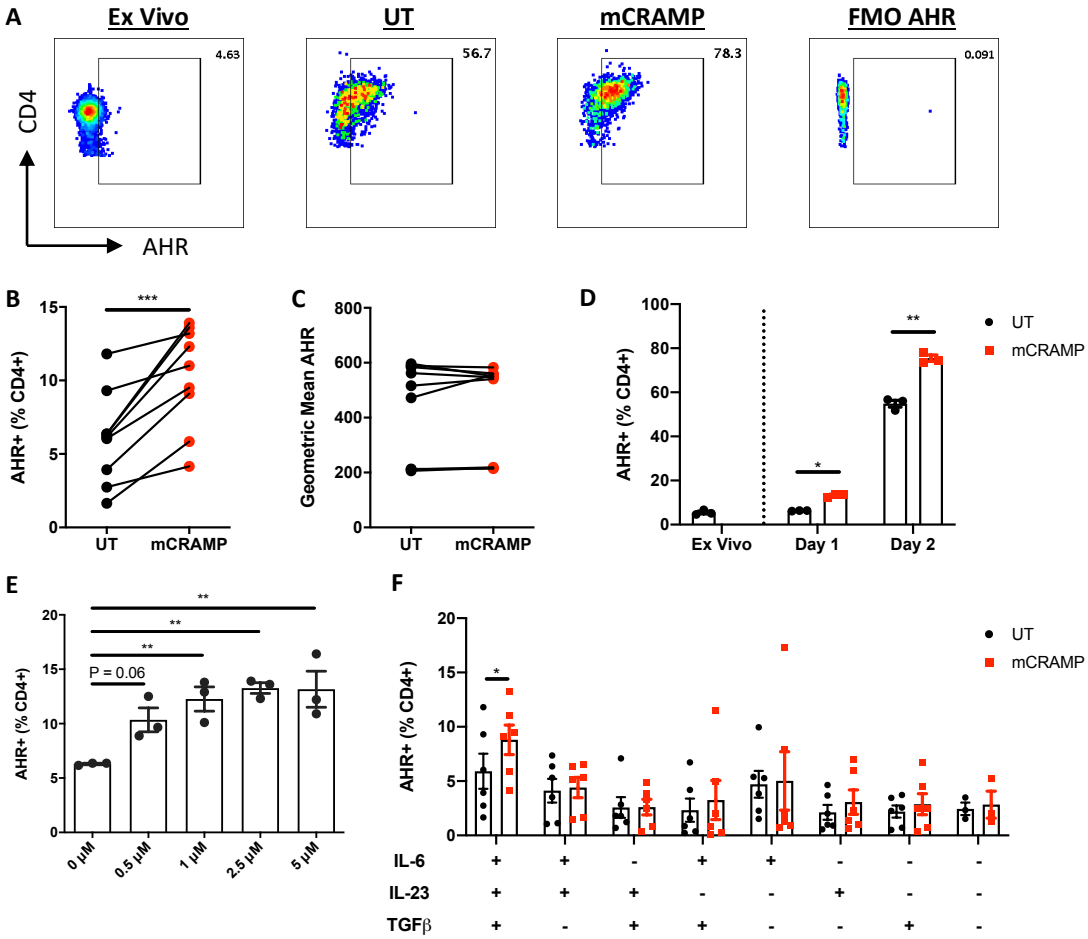


Figure 4.6: mCRAMP increases the frequency of AHR-expressing CD4⁺ T cells cultured under Th17-driving conditions. Whole single cell splenic suspensions were cultured under Th17-driving conditions, with or without 2.5 µM synthetic mCRAMP **(A)** Representative plots of AHR expression production by CD4⁺ T cells on day 1, assessed by flow cytometry **(B)** Percentages of CD4⁺ AHR⁺ T cells on day 1 (n = 9) **(C)** Geometric Mean of CD4⁺ AHR⁺ T cells on day 1 **(D)** Percentages of CD4⁺ AHR⁺ T cells, *ex vivo* and days 1-2 (n = 3) **(E)** CD4⁺ AHR⁺ vs. **m**CRAMP dose response (n = 3) **(F)** Whole splenocytes were cultured with different combinations of IL-6 (20 ng/mL), IL-23 (20 ng/mL) and TGFβ (3 ng/mL), with or without 2.5 µM synthetic mCRAMP. Percentages of CD4⁺ AHR⁺ T cells on day 1 (n = 6). Data shown is mean +/- standard error. Statistical significance (where * represents < 0.05, ** < 0.01 and *** < 0.001) was determined using a paired t-test (B) or a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test (D & F) or an ordinary one-way ANOVA with a Dunnett's multiple comparison post-test (E). UT: untreated.

4.3.4 mCRAMP acts via AHR to increase the percentage of CD4$^+$ IL-17A$^+$ IL-17F$^+$ T cells

AHR is highly expressed by Th17 lymphocytes and plays an important role in the *in vivo* and *in vitro* generation of this T helper cell subset[79]. For example, the AHR agonist FICZ (6-formylindolo[3,2-b]carbazole) increases IL-17 production by CD4$^+$ T cells cultured under Th17-driving conditions[79]. I therefore sought to determine whether the effects of mCRAMP on the differentiation of Th17 cells are dependent on the upregulation of AHR. To do this, I cultured whole single cell splenic suspensions under Th17-driving conditions for 2 days, with or without synthetic mCRAMP and/or an AHR antagonist, CH223191[447].

Figure 4.7 shows that the addition of the AHR antagonist had no effect on the percentage of IL-17A or IL-17F single-positive cells. For example, mCRAMP increased the percentage of IL-17A$^-$ IL-17F$^+$ T lymphocytes from 9.10% (+/- 0.83) to 15.76% (+/- 1.00), despite the presence of CH223191 (**Figure 4.7 B**). However, the AHR antagonist did abolish the increase in the percentage of IL-17A and IL-17F double-producing T cells normally seen in response to mCRAMP (**Figure 4.7 C**).

These results suggest that mCRAMP upregulates AHR and acts via this transcription factor to specifically promote the development of CD4$^+$ IL-17A$^+$ IL-17F$^+$ T lymphocytes.

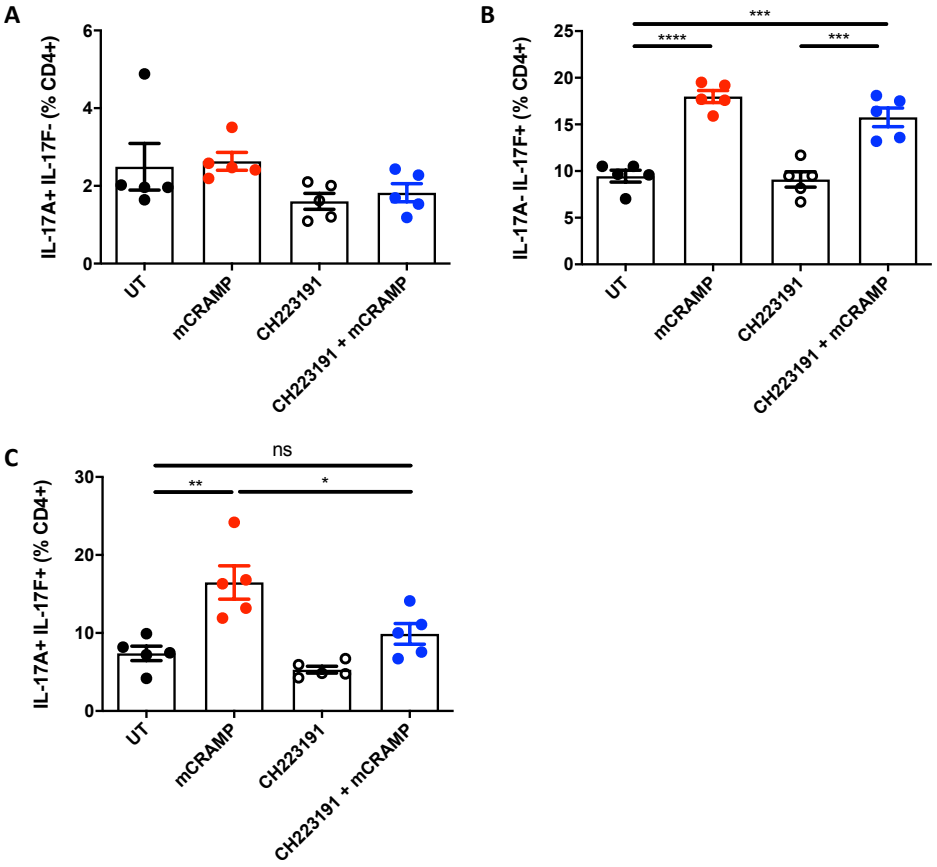


Figure 4.7: An AHR antagonist abolishes the increase in the percentage of CD4⁺ IL-17A⁺ IL-17F⁺ T cells induced by mCRAMP. Whole single cell splenic suspensions were cultured under Th17-driving conditions, with or without 2.5 μM synthetic mCRAMP and/or an AHR antagonist (CH223191, 10μM) for 2 days **(A)** Percentages of $CD4^+$ $IL\text{-}17A^+$ $IL\text{-}17F^-$ T cells on day 2 **(B)** Percentages of $CD4^+$ $IL\text{-}17A^-$ $IL\text{-}17F^+$ T cells on day 2 **(C)** Percentages of $CD4^+$ $IL\text{-}17A^+$ $IL\text{-}17F^+$ T cells on day 2. Data shown is mean +/- standard error. N = 5. Statistical significance (where * represents < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001) was determined using an ordinary one-way ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

4.3.5 mCRAMP is probably not an AHR ligand

I have shown that mCRAMP upregulated the expression of AHR by $CD4^+$ T lymphocytes, which specifically enhanced the differentiation of IL-17A and IL-17F co-expressing cells. AHR is a transcription factor that is activated by numerous environmental and endogenous ligands, which result in differential downstream effects depending on the cell type[445]. For instance, FICZ is a natural agonist which boosts Th17 differentiation, whereas TCDD, a synthetic compound found in combustion products and cigarette smoke, promotes the development of regulatory T cells *in vitro*[448].

AHR drives its own expression as part of a positive feedback loop[445]. Based on the results discussed above showing that the addition of an AHR antagonist abolished the increase in the frequency of $CD4^+$ IL-$17A^+$ IL-$17F^+$ T cells induced in response to mCRAMP, one might speculate that this peptide is a novel endogenous AHR ligand. To explore the possibility that mCRAMP can bind to the unique AHR ligand-binding domain based on its structure, I cultured whole single cell splenic suspensions under Th17-driving conditions with a variety of other host defence peptides that fold into distinctive configurations and display a net charge ranging from +3 to +6 (**Figure 4.8 A & B**). For example, "partial peptide 47" (PP47) consists of a fragment of LL-37 (the last 22 amino acids). Human β-defensin 2 (hBD2) forms a three-stranded anti-parallel β-sheet with one helix flanking the sheet[449]. Bac2A is a linear variant of the loop-shaped bactenecin that is typically found in bovine neutrophils[450]. Indolicidin is another cationic bovine antimicrobial peptide that is rich in tryptophan and proline residues[277]. I hypothesised that if mCRAMP is a novel AHR ligand, other peptides of different sequence and structure would not have the same effect on Th17 differentiation.

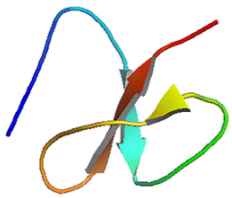
Figure 4.8 shows that PP47 significantly increased the percentage of $CD4^+$ IL-$17A^+$ T cells from 10.05% (+/- 3.05) to 17.77% (+/- 2.13). This was comparable to mCRAMP (21.60% +/- 2.00) and LL-37 (19.63% +/- 1.63). There was also a trend indicating that hBD2 increased the frequency of IL-17-producing $CD4^+$ T lymphocytes, although this was not statistically significant (16.90% +/- 1.33; P = 0.156). Conversely, addition of the bovine AMPs, Bac2A and indolicidin, led to only a very small and insignificant increase in the percentage of $CD4^+$ IL-$17A^+$ T cells: 12.92% (+/- 2.4) and 12.18% (+/- 5.01), respectively.

PP47 had similar effects on the differentiation of $CD4^+$ T cells compared to mCRAMP and LL-37. It is possible that this partial peptide has a conserved structural epitope that activates the same intracellular receptor. However, hBD2 has a very different amino acid sequence and structure compared to mCRAMP but enhanced Th17 development nonetheless. Taken together, these results suggest that it is unlikely that mCRAMP is an endogenous AHR ligand. However, further investigation will be required to confirm this.

A

Cathelicidin
(mCRAMP/LL-37)

B



Human β-defensin 2

C

Indolicidin

D

Peptide	Amino Acid Sequence	Net Charge
mCRAMP	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ	+6
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	+6
Scrambled LL-37	RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL	+6
PP47	EFKRIVQRIKDFLRNLVPRTES	+3
hBD2	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP	+6
Bac2A	RLARIVVIRVAR	+4
Indolicidin	ILPWKWPWWPWRR	+4

E

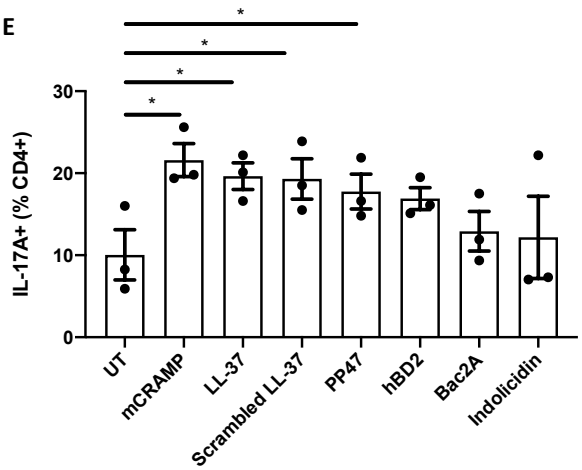


Figure 4.8: Several other peptides also increase Th17 differentiation *in vitro*. Whole single cell splenic suspensions were cultured under Th17-driving conditions with 2.5 μM synthetic mCRAMP, LL-37, Scrambled LL-37, PP47, hBD2, Bac2A or indolicidin, for 2 days **(A)** α-helical structure of cathelicidin (mCRAMP/LL-37) **(B)** Structure of human β-defensin 2 (hBD2) **(C)** Extended structure of indolicidin **(D)** Amino acid sequences and net charge of the different peptides tested **(E)** Percentages of $CD4^+$ $IL\text{-}17A^+$ T cells on day 2. Data shown is mean +/- standard error. N = 3. Statistical significance (where * represents < 0.05) was determined using an ordinary one-way ANOVA with a Dunnett's multiple comparison post-test. UT: untreated; PP47: partial peptide 47; hBD2: human β-defensin 2.

4.4 Discussion

To dissect the mechanism through which mCRAMP acts to enhance Th17 differentiation, I analysed the gene expression profile of $CD4^+$ T cells that had been cultured under Th17-driving conditions for 24 hours, with or without synthetic peptide. The results revealed that 17 genes were significantly differentially expressed (2-fold or more different).

4.4.1 mCRAMP induces genetic changes that promote T cell activation

I previously demonstrated that mCRAMP increased the activation status of $CD4^+$ T cells cultured under Th17-driving conditions (**Chapter 3: Figure 3.7**). Gene expression analysis revealed that *Sell* (CD62L) was significantly downregulated by mCRAMP, which is a hallmark of T cell activation[369]. The reduction in CD62L expression following exposure to mCRAMP is therefore in agreement with observations made previously, in which the percentage of $CD4^+$ $CD44^+$ $CD62L^-$ T cells was increased.

In addition, $CD4^+$ T lymphocytes treated with mCRAMP displayed a log2 fold change of -1.02 in *Ikbke* expression compared to untreated. IκB kinase ε promotes the phosphorylation of NFATc1 (nuclear factor of activated T cells, cytoplasmic 1), which in turn inhibits T cell responses and is therefore considered a crucial negative regulator of T cell activation[451]. Reduced expression of this gene could therefore also contribute to increasing the activation status of $CD4^+$ T cells when treated with synthetic mCRAMP.

4.4.2 mCRAMP modulates the expression of genes involved in T cell apoptosis

Several changes in the gene expression profile identified in the Nanostring data support the hypothesis that mCRAMP acts as a survival factor to increase the viability of $CD4^+$ T cells (**Chapter 3: section 3.3.10**). For example, CD9 is expressed by T cells and can deliver a potent CD28-independent co-stimulatory signal[452]. Tai and colleagues demonstrated that co-stimulation of CD9 on naïve T cells during TCR stimulation results in transient activation followed by apoptosis[453]. In the present study, CD9 was significantly downregulated in response to mCRAMP (log2 FC = -1.13). Furthermore, IL-2, whose expression displayed a log2

fold change of -1.7, participates in a regulatory feedback mechanism by predisposing mature, activated T lymphocytes to undergo activation-induced cell death[454]. The aryl hydrocarbon receptor (AHR), which was significantly upregulated by mCRAMP (log2 FC = 1.72), has also been shown to regulate gut immunity by promoting the survival of RORγt$^+$ innate lymphoid cells (ILC): absence of AHR signalling resulted in decreased levels of anti-apoptotic genes such as BCL2/BCL2L1 and increased apoptosis[455]. The changes in the expression of these genes induced by mCRAMP could therefore contribute to increasing CD4$^+$ T cell viability.

Conversely, *Fadd* was one of two genes that were significantly upregulated (2-fold or more) by mCRAMP. FADD (Fas-associated protein with death domain) is an adaptor protein that plays a crucial role in apoptosis by helping form the death-inducing signalling complex (DISC)[456]. Moreover, the anti-apoptotic gene *Bcl2* also had a log2 fold change of -0.63 in response to mCRAMP (P = 0.037; **Appendix: Table A1**). The upregulation of FADD and the concomitant downregulation of BCL2 suggest that mCRAMP promotes apoptosis. One possible explanation for these contradictory observations is that the changes in expression of *Fadd* and *Bcl2* reflect mCRAMP promoting the death of other non-Th17 subsets that I have not specifically investigated, such as Th2 and regulatory T lymphocytes. Indeed, Mader and colleagues demonstrated that human cathelicidin induces granzyme-mediated apoptosis in Tregs[360].

4.4.3 mCRAMP downregulates the expression of Th1-related genes that suppress Th17 differentiation

Several of the genes downregulated in response to mCRAMP encode classical Th1-related proteins. For instance, the expression of XCL1 was significantly decreased following exposure to mCRAMP, with a log2 fold change of -1.95. Th1 cells secrete XCL1 (lymphotactin), although unlike IFNγ, the expression of this chemokine does not require STAT4 activation[457]. The downregulation of XCL1 may therefore reflect a decrease in Th1 polarization.

Many of these Th1-related genes exert a suppressive function on the differentiation of Th17 cells. For example, BCL3 is a member of the IκB transcription factor family and has been shown to be a physiologically relevant regulator of Th1 cell plasticity[458]. More specifically,

Tang and colleagues demonstrated that the loss of BCL3 in $CD4^+$ T lymphocytes allowed for the conversion of Th1 cells into Th17 cells[458]. The authors also suggested that BCL3 stabilizes the Th1 cell phenotype, in part, by controlling the expression of the Th17 master regulator, RORγt[458]. mCRAMP may therefore increase the percentage of $CD4^+$ $IL\text{-}17^+$ T cells by downregulating BCL3 expression (log2 FC = -1.46), thereby lifting the inhibition on RORγt and promoting the conversion of Th1 lymphocytes to the Th17 lineage.

IRF8 is another transcription factor that has been heavily implicated in Th1 differentiation[459]. For instance, IRF8 has been shown to regulate the expression of IL-12, an important cytokine required for driving the development of IFNγ-producing Th1 lymphocytes[459]. IRF8-deficient mice fail to mount a protective Th1 response and as a result, develop fulminant, disseminated leishmaniasis upon infection with *L. major*[459]. In addition, Ouyang et al. demonstrated that IRF8 directs a silencing program for Th17 differentiation[440]. IRF8 KO mice exhibit enhanced Th17 polarization and the transfer of naïve T cells from these animals induces more severe colitis in $RAG^{-/-}$ mice[440]. Moreover, IRF8 suppresses the expression of IL-17 by physically interacting with RORγt[440]. mCRAMP induced a log2 fold change of -1.44 in IRF8 expression. Downregulation of IRF8 by this peptide may therefore contribute to increasing Th17 differentiation by preventing the silencing program normally induced by this transcription factor from taking effect.

Another Th1-related protein downregulated by mCRAMP was IKZF4/EOS, a member of the Ikaros family of transcription factors (log2 FC = -1.49). EOS is highly expressed in Th1 cells[460]. Furthermore, Rieder and colleagues found that $EOS^{-/-}$ mice developed more severe EAE, characterized by increased effector T cells in the periphery and CNS, as well as amplified IL-17 production[461]. The exact mechanism through which EOS suppresses Th17 differentiation remains unknown. However, this transcription factor is required for IL-2 production by $CD4^+$ T lymphocytes *in vitro*[461]. IL-2 is an important growth factor that promotes the development of Th1, Th2 and cytotoxic $CD8^+$ T cells[441]. For example, IL-2 is essential for the acquisition of effector functions mediated by IFNγ[441]. It has also been shown to strongly antagonize Th17 differentiation: Laurence et al. demonstrated that the addition of IL-2 to Th17 cultures led to a dose-dependent decrease in RORγt expression and proportion of cells producing IL-17[442]. This was mediated by STAT5, which can directly bind to the *Il17* promoter to suppress its

transcription[442]. Nanostring analysis revealed that mCRAMP significantly downregulated the expression of *Il2* (log2 FC = -1.7). Furthermore, one of the most important effects of TGFβ on T cells is the suppression of IL-2 production[462]. I have previously hypothesised that mCRAMP enhances TGFβ signals to enhance Th17 differentiation. mCRAMP may therefore promote the development of IL-17-producing $CD4^+$ T lymphocytes *in vitro* by relieving the inhibition of IL-2 on Th17 differentiation.

Taken together, the Nanostring data suggests that mCRAMP skews T helper cell differentiation away from the Th1 lineage and enhances Th17 polarization by downregulating the expression of classical Th1-related genes that also suppress the development of Th17 lymphocytes.

4.4.4 mCRAMP downregulates the expression of genes involved in the negative regulation of Th17 differentiation

SOCS3 expression was significantly downregulated by $CD4^+$ T cells in response to mCRAMP (log2 FC = -1.04). This "suppressor of cytokine signalling" is a negative modulator of STAT3 phosphorylation and Th17 differentiation[60]. For instance, SOCS3 deficiency has no effect on the differentiation of Th1 or Th2 T helper subsets but IL-23-induced STAT3 phosphorylation is enhanced in its absence[60]. STAT3 directly binds to the promoter regions of IL-17A and IL-17F, thereby promoting their transcription[60]. Signalling through STAT3 therefore plays an important role in Th17 development and persistent STAT3 phosphorylation has been associated with autoimmune inflammation[463]. Over-expression of SOCS3 in T cells results in reduced STAT3 phosphorylation, decreased Th17 differentiation and significantly delayed EAE onset[464]. TGFβ inhibits IL-6 and IL-21-induced SOCS3, thereby enhancing, as well as prolonging, STAT3 activation in naïve $CD4^+$ T cells[66]. My results suggest that mCRAMP requires TGFβ to amplify Th17 responses. It is therefore possible that mCRAMP achieves this by synergizing with TGFβ to further downregulate SOCS3 and relieve its inhibitory effects.

Similarly, *Tcf7* had a log2 fold change of -1.12 in response to treatment with mCRAMP. *Tcf7* encodes TCF1, which negatively regulates the expression of the IL-17 family of cytokines, in part, by binding to the regulatory regions of the *Il17* gene[439]. Moreover, Yu et al. found that

Th17 cells from TCF1-deficient mice express high levels of IL-7Rα, which promotes their survival and expansion *in vivo*[439]. TCF1 is therefore considered a critical negative regulator of the inflammatory potential of TCR-activated T cells and autoimmunity[439]. Downregulation of *Tcf7* expression is consequently another way in which mCRAMP can enhance Th17 differentiation, by removing a layer of negative regulation and promoting the survival of $CD4^+$ $IL\text{-}17^+$ T cells.

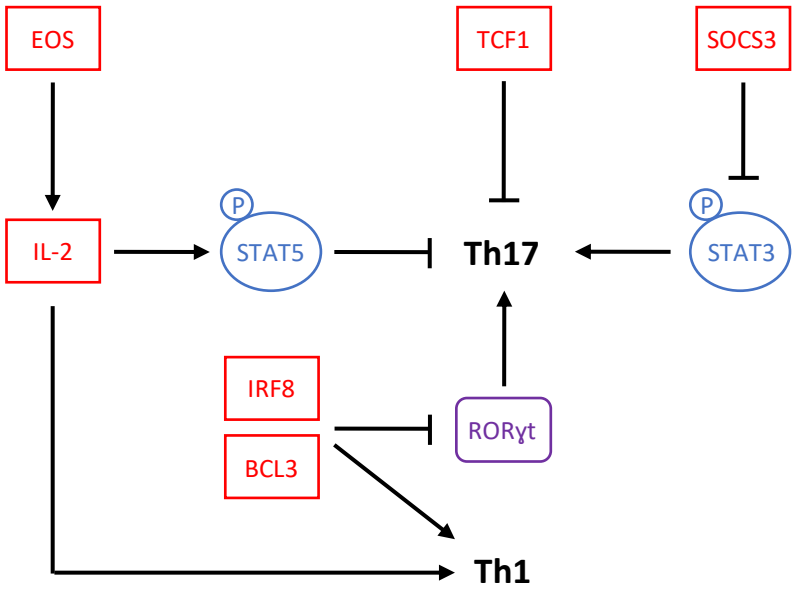


Figure 4.9: The regulatory networks downregulated by mCRAMP. Nanostring analysis revealed that the expression of genes in red was significantly downregulated in $CD4^+$ T cells cultured under Th17-driving conditions following exposure to synthetic mCRAMP. IRF8 and BCL3 are classical Th1-related genes, both of which also target the RORγt transcription factor to silence the Th17 differentiation program. EOS is required for the production of IL-2, a potent Th17 antagonist, which acts via STAT5 to inhibit the expression of IL-17. TCF1 and SOCS3 are negative regulators of the Th17 response. SOCS3 controls the phosphorylation of STAT3, which drives Th17 polarization.

4.4.5 mCRAMP skews T helper cell differentiation away from the Th1 lineage

mCRAMP downregulated the expression of genes classically associated with the Th1 lineage when the cells were cultured under Th17-driving conditions. Furthermore, I found that the percentage of $CD4^+$ T lymphocytes expressing the Th1 master transcription factor, T-bet, was significantly reduced by mCRAMP.

Master regulators, such as T-bet, shape immune responses by activating one genetic program whilst simultaneously silencing the activity of factors that drive the differentiation of other subsets[465,466]. For example, T-bet suppresses the generation of Th2 cells by blocking the expression of IL-4 and interfering with GATA3[30,467]. Lazarevic and colleagues demonstrated that T-bet also inhibits the development of the Th17 lineage by interacting with RUNX1 to block the transactivation of the *Rorc* promoter[466]. Ectopic T-bet expression in both naïve $CD4^+$ T cells and committed Th17 cells is sufficient to repress the expression of RORγt and IL-17 production[466]. The decrease in T-bet expression induced by mCRAMP therefore supports the hypothesis that this host defence peptide skews T helper cell differentiation away from the Th1 lineage and in doing so, promotes Th17 development.

Th1 lymphocytes are the primary producers of IFNγ[26]. mCRAMP significantly decreased the percentage of $CD4^+$ $IFN\gamma^+$ T cells in Th17 cultures, reflecting the downregulation of T-bet and the skewing of T helper cell differentiation. Interestingly, these IFNγ-producing $CD4^+$ T lymphocytes were not protected from cell death, unlike $CD4^+$ $IL\text{-}17A^+$ T cells. This could therefore account for the reduced frequency of this T helper subset.

Similarly to T-bet, IFNγ also exerts suppressive effects on Th17 development[468]. More specifically, Yeh et al. reported that IFNγ signals through STAT1 to inhibit the production of IL-17A and IL-17F in a T-bet-independent manner[468]. The suppression of Th1 polarization and subsequent decrease in IFNγ production therefore highlights another potential mechanism through which mCRAMP increases Th17 potential.

TGFβ is a known suppressor of T-bet expression and I have previously shown that mCRAMP requires TGFβ to amplify Th17 responses (**Chapter 3: Figure 3.19**)[469]. mCRAMP did not have any effect on IFNγ production when the cells were cultured under non-lineage- (**Chapter 3: Figure 3.5**) or Th1-driving conditions (**Chapter 3: Figure 3.18**), which could be explained by the absence of TGFβ in these cultures. Taken together, this supports the hypothesis that mCRAMP skews T helper cell differentiation away from the Th1 lineage and promotes Th17 development in a TGFβ-dependent manner.

T-bet is not only essential for driving Th1 development[465]. It is also required for the differentiation and function of specialized effector populations that arise from other lymphocyte lineages[443]. For instance, Th17 cells can co-express T-bet and RORγt to promote their functional diversification[443]. The chromatin conformation of the *Tbx21* locus remains in a transcriptionally poised state in differentiated Th17 cells, which partially accounts for the plasticity of this T helper subset[470]. RORγt/T-bet double-positive cells have been found in lesional tissue in EAE, as well as in patients with multiple sclerosis[444,471]. Interestingly, they represent a higher proportion of infiltrating Th17 cells in the CNS compared to RORγt single-positive lymphocytes[444]. Moreover, T-bet-mediated acquisition of IFNγ expression promotes the accumulation of encephalitogenic Th17 cells in the CNS and tissue inflammation during EAE[443,472]. It has therefore been suggested that secondary expression of T-bet is required to stabilize pathogenic IL-17 and IFNγ double-producing Th17 lymphocytes, which correlate with disease severity[443,444]. In the present study, I did not detect any $CD4^+$ IL-$17A^+$ $IFN\gamma^+$ T cells *in vitro* but a substantial proportion of $CD4^+$ T lymphocytes expressed both RORγt and T-bet. Furthermore, the addition of mCRAMP appeared to decrease their frequency following 3 days of culture. The physiological relevance of this is unclear but could provide further evidence to support the hypothesis that mCRAMP reduces the pathogenic potential of this T helper cell subset.

4.4.6 mCRAMP upregulates the aryl hydrocarbon receptor

Gene expression analysis revealed that mCRAMP significantly upregulated the expression of the aryl hydrocarbon receptor (AHR) in $CD4^+$ T cells cultured under Th17-driving conditions. AHR is ligand-activated transcription factor that responds to a variety of environmental stimuli derived from the diet, commensal flora and host metabolism[445]. AHR expression is driven by the direct transactivation of the AHR promoter by phosphorylated STAT3, which is induced by IL-6 and IL-21 during Th17 differentiation[445,473].

AHR is kept in an inactive state as part of a protein complex, which stabilizes it in the cytoplasm and helps it maintain a conformation that has high affinity for its ligands[445]. Upon activation, AHR undergoes a conformational change that exposes the AHR amino-terminal nuclear localization and export signal, resulting in its translocation to the nucleus[445]. AHR

controls target gene expression by dimerizing with ARNT, which is recruited to xenobiotic response elements (XREs)[445].

AHR plays an important role in Th17 development[446]. For example, several Th17-related genes harbour XREs, including IL-17A, IL-17F, IL-22 and RORγt[79]. $CD4^+$ T lymphocytes from AHR-deficient mice can differentiate into Th17 cells but display impaired production of IL-17 and undetectable IL-22[79]. In line with attenuated Th17 differentiation *in vitro*, AHR knockouts develop a much milder form of EAE with delayed kinetics[79].

Exposure of $CD4^+$ T cells to the AHR agonist, FICZ, under Th17-polarizing conditions strongly enhances IL-17A, IL-17F and IL-22 production[79]. However, the downstream effects of AHR are ligand-specific[448]. Quintana et al. found that binding of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) to AHR suppressed Th17-mediated autoimmunity and EAE by promoting the development of $FOXP3^+$ regulatory T cells, whereas FICZ enhanced the disease by stimulating the generation of Th17 lymphocytes[79,448]. Similarly, administration of ITE (2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester), another tryptophan-derived AHR endogenous ligand, also resulted in reduced EAE severity[448].

Conversely, Duarte et al. demonstrated that both FICZ and TCDD upregulate Th17 development *in vitro*[474]. The authors suggested that the discrepancy between their work and previously published studies is due to the fact that the effects of AHR ligands depend on timing and that their mode of action *in vivo* is likely shaped by their differential susceptibility to metabolic feedback control[474]. For example, FICZ is an ultraviolet photoproduct of tryptophan synthesized *in vivo* that is rapidly metabolized, thus inducing only transient AHR signalling, whereas TCDD is the most stable of all xenobiotic AHR ligands[474]. They concluded that the high toxicity of TCDD could cause a proportional shift in the numbers of Tregs due to the death of other cell types, rather than an actual expansion of this subset[474].

I have shown that mCRAMP increased the percentage of $CD4^+$ $IL\text{-}17F^+$ and IL-17A/IL-17F double-producing cells in Th17 cultures. Furthermore, mCRAMP significantly increased the expression of AHR. Consistent with its established role in promoting the development of Th17 lymphocytes, addition of an AHR antagonist (CH223191) abolished the increase in the

percentage of $CD4^+$ IL-17A^+ Il-17F^+ T cells normally induced by mCRAMP. However, it had no effect on the proportions of IL-17A and IL-17F single-positive lymphocytes. Once again, this implies that there are at least two pathways induced by mCRAMP, one of which is AHR-dependent. Wanke et al. suggested that the IL-17A and IL-17F double-producers represent a regulatory subset of Th17 cells known to be induced in response to TGFβ[398,403]. Furthermore, Alves de Lima and colleagues demonstrated that TGFβ signalling sustains the expression of AHR in $CD4^+$ T cells[475]. I have shown that mCRAMP requires TGFβ in order to boost Th17 differentiation (**Chapter 3: Figure 3.19**). It is therefore possible that mCRAMP specifically upregulates AHR expression, in concert with TGFβ, in this particular Th17 subset to increase both IL-17A and IL-17F production.

AHR can control the expression of genes that do not harbour XREs to promote Th17 differentiation (**Figure 4.10**)[445]. For instance, AHR interacts with STAT1 and STAT5 (but not STAT3 or STAT6) and subsequently modulates their activity[81]. IFNγ-induced STAT1 activation inhibits Th17 differentiation by binding to the *Il17* promoter and suppressing its expression[476]. Whilst STAT3 remains active under Th17-culture conditions, STAT1 activation is relatively transient and returns to basal levels within 24 hours[81]. The maintenance of its activation can therefore prevent the interaction between RORγt and *Il17* by masking their binding sites[81]. Kimura et al. demonstrated that STAT1 activation remains activated at 24 hours after stimulation in naïve $CD4^+$ T cells from AHR-deficient mice[81]. AHR has dual functions in controlling intracellular protein levels: it serves both as a transcription factor to promote the transcription of target genes, as well as a ligand-dependent E3 ubiquitin ligase that regulates selective protein degradation[477]. The authors therefore suggested that AHR targets activated STAT1 for degradation, thereby relieving its inhibition on Th17 differentiation[81]. Indeed, the Nanostring data generated in this study revealed that STAT1 was significantly downregulated (log2 FC = -0.69, P = 0.005). The upregulation of AHR induced by mCRAMP could consequently represent a mechanism through which it suppresses STAT1 activation to promote the development of IL-17-producing $CD4^+$ T lymphocytes.

In addition to modulating STAT1 activation, AHR indirectly promotes Th17 differentiation by regulating the inhibitory effects of IL-2, which interferes with the development of this T helper cell subset through the activation of STAT5[442]. More specifically, AHR cooperates with

STAT3 to induce the expression of AIOLOS (IKZF3), another member of the Ikaros transcription factor family[478]. AIOLOS binds to the IL-2 promoter, inducing chromatin modifications that result in IL-2 silencing both *in vitro* and *in vivo*[478]. No significant differences in *Ikzf3* expression were detected between untreated and mCRAMP-treated samples in the Nanostring data generated for this study. However, IL-2 was significantly downregulated (log2 FC = -1.7) and an almost complete suppression of production was observed by ELISA. It is therefore possible that mCRAMP increases the percentage of $CD4^+$ $IL\text{-}17A^+$ $IL\text{-}17F^+$ T cells in an AHR-dependent manner by reducing the expression of IL-2, thereby preventing it from exerting its antagonistic effects.

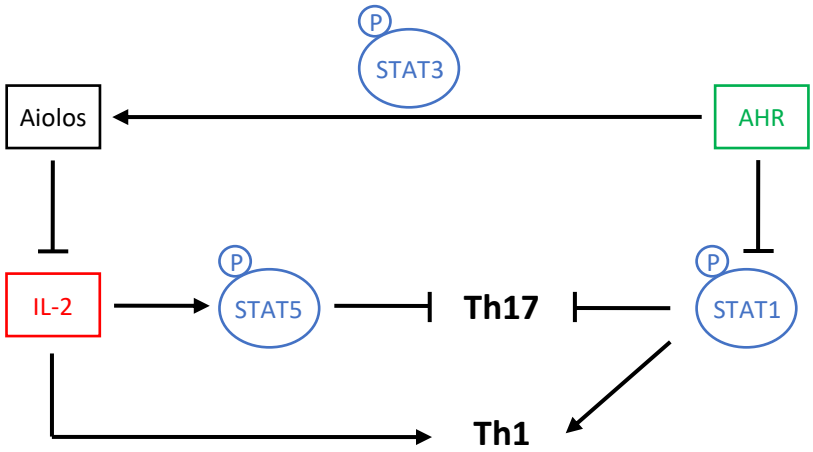


Figure 4.10 AHR and its role in Th17 development. AHR was significantly upregulated by CRAMP (green). AHR targets STAT1 for degradation, thereby relieving its inhibition on Th17 differentiation. AHR cooperates with STAT3 to induce the expression of Aiolos. Aiolos binds to the IL-2 promoter, inducing chromatin modifications that result in IL-2 silencing. IL-2 is a Th17 antagonist, which acts via STAT5 to inhibit the expression of IL-17.

AHR has been heavily implicated in the production of IL-22 by Th17 lymphocytes[79]. Naïve $CD4^+$ T cells from AHR-deficient mice cultured under Th17-driving conditions cannot produce IL-22[79]. RORγt and STAT3 facilitate the recruitment of AHR to the *Il22* promoter and activation of this transcription factor by FICZ promotes the expression of the cytokine[79,111]. Although mCRAMP significantly upregulated AHR, the peptide had no effect on IL-22 production by $CD4^+$ T cells in Th17 cultures (**Chapter 3: Figure 3.9**). Several groups have suggested that AHR activates distinct pathways to promote IL-17 and IL-22 production by Th17 lymphocytes[475].

Based on my results, mCRAMP appears to only influence the pathway that is responsible for driving IL-17 expression.

4.4.7 TGFβ is not sufficient for mCRAMP to increase AHR expression

I have provided evidence that suggests mCRAMP requires TGFβ to boost Th17 differentiation (**Chapter 3: Figure 3.19**). However, the upregulation of AHR does not: mCRAMP increased the percentage of $CD4^+$ AHR^+ T cells only when all three Th17-polarizing cytokines were present. TGFβ is a relatively poor inducer of *AHR* alone and IL-6 downstream signalling plays an important synergistic role that drives its expression[475]. It is also possible that mCRAMP increases AHR expression indirectly. For example, mCRAMP could induce cellular changes that modulate tryptophan metabolism and the production of endogenous AHR ligands, which in turn promote AHR expression as part of a positive feedback loop[445].

4.4.8 mCRAMP reduces the pathogenic potential of Th17 cells

AHR has been described as a marker of non-pathogenic Th17 cells[475]. As discussed previously, the cytokine milieu present during the differentiation process determines whether naïve T cells become pathogenic or not[479]. For example, treatment of naïve $CD4^+$ T cells with TGFβ and IL-6 is generally thought to generate a non-pathogenic population of Th17 lymphocytes: adoptive transfer of these cells does not typically induce EAE[479,480]. However, the addition of IL-23 abrogates this and drives their conversion to a more pathogenic phenotype[402,479]. Ghoreschi and colleagues demonstrated that AHR expression was observed only in non-pathogenic "conventional" Th17 cells (generated with TGFβ) and not in those cultured with IL-23[444,446]. The upregulation of this transcription factor in response to mCRAMP may therefore suggest that the peptide drives the development of a non-pathogenic population of Th17 lymphocytes.

Several studies have tried to identify the transcriptional signature of pathogenic and non-pathogenic Th17 cells by comparing gene expression profiles of *in vitro*-generated Th17 lymphocytes, as well as those induced *in vivo* (such as during EAE)[402,479,481]. Pathogenic Th17 cells express more effector molecules, including pro-inflammatory cytokines/chemokines

such as CXCR3, CCL4, IL-2 and IL-22. mCRAMP had no effect on IL-22 production (**Chapter 3: Figure 3.9**) but the Nanostring data revealed a significant downregulation of IL-2, which was confirmed by ELISA. Moreover, the expression of CXCR3, which is important for trafficking of T cells to sites of inflammation, was reduced in $CD4^+$ T cells cultured under Th17-driving conditions and in the presence of mCRAMP (log2 FC = -0.672, P = 0.032; **Appendix: Table A1**). Similarly, CCL4 was also downregulated, although this was not statistically significant (log2 FC = -1.86, P = 0.399; **Appendix Table A1**).

Pathogenic Th17 lymphocytes also upregulate the expression of T-bet and HLX1, another Th1-related transcription factor that induces IFNγ production[482]. Yang et al. demonstrated that T-bet was essential for the encephalitogenicity of both Th1 and Th17 cells during EAE[483]. I have shown that mCRAMP decreases the percentage of $CD4^+$ T-bet^+ T lymphocytes, as well as those co-expressing RORγt and T-bet.

On the other hand, non-pathogenic Th17 cells upregulate the expression of molecules typically associated with immune suppression, including IL-10, AHR and c-MAF[402,479]. AHR physically interacts with c-MAF to promote the transactivation of the *Il10* promoter, which is essential for the development of IL-10-producing regulatory T cells[484]. Furthermore, AHR has been shown to mediate the conversion of Th17 cells into IL-10^+ anti-inflammatory Tregs if lacking pathogenicity signals[420]. In the present study, *Maf* was significantly upregulated by mCRAMP (log2 FC = 0.847, P = 0.004; **Appendix: Table A1**). IL-10 was also increased across all three samples but was excluded from the analysis due to low counts.

Taken together, these results suggest that mCRAMP downregulates the expression of various pro-inflammatory molecules, whilst simultaneously upregulating those involved in immune regulation, to reduce the pathogenic potential of developing Th17 cells.

4.4.9 mCRAMP is probably not an AHR ligand

Based on the results generated throughout this study, one might speculate as to whether mCRAMP constitutes an as of yet unidentified endogenous AHR ligand. However, I have shown that the D-enantiomer and scrambled peptide increased Th17 differentiation to the

same extent as mCRAMP. Moreover, both PP47 (a partial LL-37 peptide) and hBD2 also enhanced the development of IL-17-producing $CD4^+$ T lymphocytes in Th17 cultures. It is highly unlikely that all six peptides are AHR ligands based on their structural differences and amino acid sequences.

Amphipathicity is important for antimicrobial activity[485]. An amphipathic helix is defined as an α helix with opposing polar (hydrophilic) and non-polar (hydrophobic) faces[486]. mCRAMP, LL-37 and D-LL-37 all fold into a characteristic amphipathic α-helical structure[277]. hBD2 and PP47 also both display amphipathic properties: hBD2 possesses an α-helical segment and despite being a much shorter peptide (22 amino acids), PP47 retains a hydrophobic face[487]. On the other hand, Bac2A is a linear peptide that cannot spontaneously fold into this helical conformation in solution[450]. Furthermore, indolicidin displays an extended structure due to the predominance of tryptophan and proline residues[488]. It is therefore possible that the amphipathicity of mCRAMP, LL-37, D-LL-37, PP47 and hBD2 is responsible for their immunomodulatory effects on Th17 differentiation. For instance, amphipathic topology is essential for the insertion of antimicrobial peptides into biological membranes[485]. As discussed previously, cathelicidin has been shown to indirectly activate P2X7R by interacting with the cytoplasmic C' terminal end of the receptor following membrane insertion[326]. mCRAMP and similar amphipathic peptides might amplify Th17 differentiation by non-specifically transactivating a yet unknown intracellular or transmembrane receptor. In addition, this could also provide a route that allows for the non-specific uptake of the peptide into the cell, similar to how it transfers extracellular DNA plasmids to the nuclear compartment via lipid rafts and proteoglycan-dependent endocytosis[333].

However, scrambled LL-37, which is made up of the same amino acids but in a random order, maintains its net positive charge but loses its propensity to fold into an amphipathic helical structure, according to its helical wheel. Despite this, scrambled LL-37 was still capable of enhancing Th17 differentiation and to the same extent as LL-37 and mCRAMP. One possible explanation for this is that our stocks of this particular peptide contain a contaminant from the manufacturing process or low levels of LPS (although it was tested for endotoxin contamination at the beginning of the project). On the other hand, it could simply suggest that the effects of mCRAMP on Th17 polarization may in fact not be due to its amphipathic

nature. I previously postulated that perhaps mCRAMP was non-specifically activating an unknown receptor by charge (**Chapter 3: section 3.4.4**). mCRAMP, LL-37, D-LL-37, scrambled LL-37 and hBD2 all display a net charge of +6. PP47 is only +3, although it could be argued that this is still quite positive. As a result, this potential mechanism of action should not be disregarded and could be tested in the future by examining the effects of other short positive peptides of random sequence and structure.

4.5 Summary

To summarize, I have shown that mCRAMP significantly downregulates the expression of Th1-related genes in $CD4^+$ T lymphocytes cultured under Th17-driving conditions, as well as those that are involved in the negative regulation of Th17 responses. In accordance with this, mCRAMP significantly decreases the frequency of T-bet-expressing $CD4^+$ T cells and IFNγ production, suggesting that the peptide skews T helper cell differentiation away from the Th1 lineage to promote the development of IL-17-producing lymphocytes.

Expression of the aryl hydrocarbon receptor was significantly increased by mCRAMP. AHR has been heavily implicated in Th17 differentiation and I have shown that the increase in the percentage of $CD4^+$ IL-$17A^+$ IL-$17F^+$ T cells induced by mCRAMP is AHR-dependent. However, an AHR antagonist had no effect on the frequency of IL-17F single-positive cells, indicating that there are at least two pathways induced by the peptide, one of which is AHR-dependent and one which is AHR-independent.

CHAPTER 5

Lymph node neutrophils may be the cellular source of mCRAMP that amplifies Th17 responses *in vivo*

5.1 Introduction

Cathelicidin is produced by a variety of cell types, including circulating neutrophils, epithelial cells, myeloid bone marrow cells, keratinocytes, monocytes and macrophages[277,281]. Transcripts of the *CAMP* gene, as well as hCAP-18 protein, have also been detected in B and T lymphocytes, as well as in natural killer (NK) cells, but in significantly lower quantities[277,352,489]. For instance, Kin et al. demonstrated that all mouse B cell subsets, as well as $CD4^+$ and $CD8^+$ T lymphocytes, produce *Camp* mRNA[489]. However, these findings were in contrast to those published by Agerberth and colleagues, who used RT-PCR and immunohistochemical staining to show that $CD3^+$ T cells do not express cathelicidin[352]. Furthermore, whether or not the protein is processed to its biologically active form remains to be verified. For example, neutrophil-derived cathelicidin is proteolytically processed by proteinase-3, which is released together with cathelicidin during degranulation[289]. Indeed, Kin and colleagues only detected immature cathelicidin in B and T lymphocytes[489]. The physiological significance of cathelicidin expression by these cells is therefore unknown.

There is an abundance of evidence that suggests there is bidirectional cross-talk between neutrophils and Th17 cells. For instance, Th17 cells produce neutrophil chemoattractants such as CXCL8[490]. Furthermore, IL-17A and IL-17F increase the release of G-CSF and CXCL8 by epithelial cells, which promotes neutrophil migration and activation[491]. On the other hand, neutrophils recruit Th17 lymphocytes to sites of inflammation through the production of CCL20/CCL2[490]. Thewissen et al. found that neutrophils dose-dependently increased IFNγ and IL-17 production by $CD4^+$ T cells in an *in vitro* co-culture model[492]. In addition, neutrophils have been shown to assist in the induction of Th17-specific responses during vaccination against *Mycobacterium tuberculosis*: neutrophil depletion abrogated Th17 induction in the lungs and spleen following mc^2-CMX vaccination[493]. Neutrophil cytoplasts, the remnants of neutrophil extracellular traps (NETs) following expulsion of their nuclear DNA, also induce Th17 differentiation in severe asthma[233].

Neutrophils play an important role in several chronic inflammatory and autoimmune disorders in which the pathogenesis is driven by Th17 lymphocytes. For example, NETs induce the generation of human $CD3^+$ $CD4^+$ IL-17^+ T cells and have subsequently been implicated in the development of psoriasis[494]. Moreover, neutrophils migrate to the articular cavity during

the early stages of rheumatoid arthritis (RA), where they become activated and are prone to NETosis[495]. Cathelicidin is displayed extracellularly on the surface of NETs and elevated levels of cathelicidin have been detected in the inflamed synovium and psoriatic epidermis[284,365,496]. Based on my previous results, I hypothesised that neutrophils are the cellular source of cathelicidin (mCRAMP) that amplifies Th17 responses *in vivo* following inoculation with heat-killed *S. typhimurium* (HKST).

5.2 Aims

Historical data generated by the laboratory demonstrated that mCRAMP-deficient mice could not produce IL-17 following inoculation with HKST (**Chapter 1: Figure 1.10**). I have shown that mCRAMP promotes Th17 differentiation *in vitro*, in part by enhancing TGFβ signals and by upregulating the aryl hydrocarbon receptor. I therefore sought to identify the cellular source of mCRAMP that is responsible for enhancing the development of IL-17-producing $CD4^+$ T cells and the location where T lymphocytes sense this host defence peptide *in vivo* during inflammation induced by HKST.

5.3 Results

5.3.1 mCRAMP does not boost Th17 differentiation of previously activated $CD4^+$ T cells

Antigen-presenting cells (APCs) collect antigen in the peripheral tissues and migrate to the draining lymph nodes where they present antigen peptides in complex with MHC molecules to naïve T cells[497]. Naïve T cells recognise antigen-MHC complexes via their TCR and become activated upon co-stimulation by mature DCs[497]. Primed T cells then migrate back to the tissue via the lymphatic system or bloodstream, where they receive additional signals and become fully differentiated (**Figure 5.1 A**)[497].

Naïve T cells spend up to 24 hours surveying an individual lymph node for their cognate antigen and receiving activation signals from DCs[498,499]. Miller and colleagues demonstrated that 80% of antigen-specific T cells within the lymph node were activated (expressed CD69)

by 24 hours following immunisation with OVA^{499}. I have shown that the addition of synthetic mCRAMP to Th17 cultures on day 0 increased the frequency of $CD4^+$ $IL\text{-}17^+$ T cells (**Chapter 3: Figure 3.8**). This is a model of naïve T lymphocytes sensing mCRAMP in the lymph node, at the same time as they are being activated by APCs.

To determine whether mCRAMP also enhanced Th17 differentiation of previously activated or primed T cells, I cultured whole single cell splenic suspensions under Th17-driving conditions and compared the effects of the peptide when added on day 0 and day 1 (**Figure 5.1**). Figure 5.1 B shows that in my Th17 cultures, there was a significant increase in the percentage of activated $CD4^+$ $CD62L^-$ $CD44^+$ T cells on day 1 (46.27% +/- 3.80) compared to day 0 (14.63% +/- 2.06). Based on this increase in T cell activation, the addition of mCRAMP on day 1 is therefore a model of them sensing the peptide after having received primary and co-stimulatory signals in the lymph node and having potentially arrived at the site of inflammation.

The addition of mCRAMP on day 0 or day 1 had no effect on the percentage of IL-17A single-positive T cells. On the other hand, mCRAMP also failed to increase the proportions of $CD4^+$ $IL\text{-}17F^+$ or IL-17A and IL-17F double-producing lymphocytes when added on day 1. For example, the addition of mCRAMP on day 0 increased the percentage of $CD4^+$ $IL\text{-}17A^+$ $IL\text{-}17F^+$ T cells from 6.78% (+/- 1.30) to 17.13% (+/- 0.60) (**Figure 5.1 E**). However, when added on day 1, this rose non-significantly to only 9.46% (+/- 1.43).

These results indicate that mCRAMP acts specifically on naïve T lymphocytes and not on those that have already been activated or fully differentiated T cells.

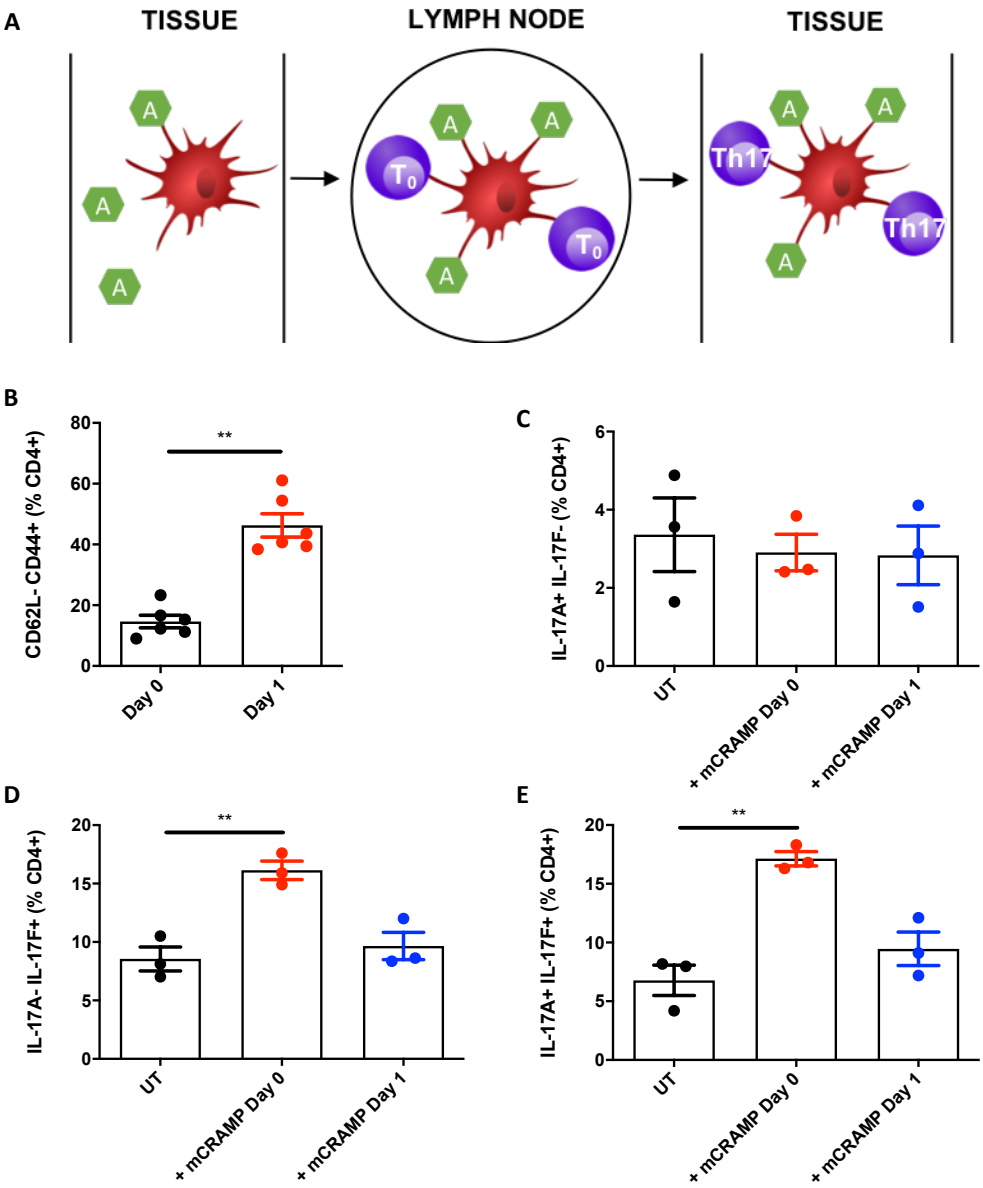


Figure 5.1: mCRAMP boosts IL-17 production of naïve but not activated T cells. (A) T cell activation and differentiation: antigen-presenting cells collect antigen in the peripheral tissues and migrate to the draining lymph nodes where they present antigen peptides to naïve T cells. Following activation, primed T cells migrate back to the tissue, where they receive additional signals and become fully differentiated **(B)** Whole single cell splenic suspensions were cultured under Th17-driving conditions for 2 days. 2.5 µM synthetic mCRAMP was added on day 0 or day 1. Percentages of activated $CD4^+$ $CD62L^-$ $CD44^+$ T cells on day 0 and day 1 confirm T cell activation **(C)** Percentages of $CD4^+$ $IL\text{-}17A^+$ $IL\text{-}17F^-$ T cells on day 2 **(D)** Percentages of $CD4^+$ $IL\text{-}17A^-$ $IL\text{-}17F^+$ T cells on day 2 **(E)** Percentages of $CD4^+$ $IL\text{-}17A^+$ $IL\text{-}17F^+$ T cells on day 2. Data shown is mean +/- standard error. N = 3. Statistical significance (where ** represents < 0.01) was determined using an ordinary one-way ANOVA with a Dunnett's multiple comparison post-test. A: antigen; UT: untreated.

5.3.2 mCRAMP at the site of inflammation does not enhance Th17 differentiation

The data discussed above suggests that naïve $CD4^+$ T cells must sense mCRAMP within the first 24 hours of activation in order to boost Th17 differentiation, implying that the effects seen *in vivo* are mediated in the lymph nodes. To test this hypothesis, I carried out an *in vivo* model in which the peptide was applied directly to the site of inflammation. T cells would therefore only come in to contact with the synthetic peptide after having been activated and recruited to the tissue. I therefore hypothesised that the frequency of $CD4^+$ IL-17^+ T lymphocytes would not be increased in this setting.

The topical application of Aldara cream (containing 5% imiquimod (IMQ)) to mouse skin causes cutaneous inflammation[500]. IMQ activates TLR7/8, which is expressed by monocytes, macrophages and plasmacytoid DCs (pDCs)[500,501]. This leads to the enhanced migration of Langerhans cells from the treated skin to the draining lymph nodes, where they activate T lymphocytes[502,503]. The ensuing immune response promotes epidermal hyperplasia, leukocyte infiltration and the development of skin lesions resembling those found in human psoriasis[500]. The exact mechanisms that drive IMQ-induced skin inflammation are yet to be fully defined[504]. However, IL-17-producing Th17 cells have been shown to play a crucial role[500].

Aldara was applied daily to the ears of WT mice, with or without synthetic mCRAMP (10 μg/mouse). The ears were collected and digested on day 3 for phenotypic analysis by flow cytometry (**Figure 5.2**). Day 3 was chosen because the first signs of inflammation are typically observed 2 or 3 days after the start of IMQ treatment (e.g. erythema, scaling, thickening) and Horvath and colleagues identified T cell infiltration in the dermis at this time point[500,505].

Figure 5.2 shows that mCRAMP did not affect the proportions of $CD4^+$ RORγt^+ or IL-$17A^+$ T cells recruited to the ears following treatment with 5% IMQ. Assuming that the peptide is not transported to the draining auricular lymph nodes, this suggests that mCRAMP present in the inflamed tissue is not sufficient to enhance Th17 polarization of previously activated $CD4^+$ T lymphocytes. These results therefore support the hypothesis that mCRAMP is required during the first 24 hours of T cell activation in order to promote Th17 differentiation.

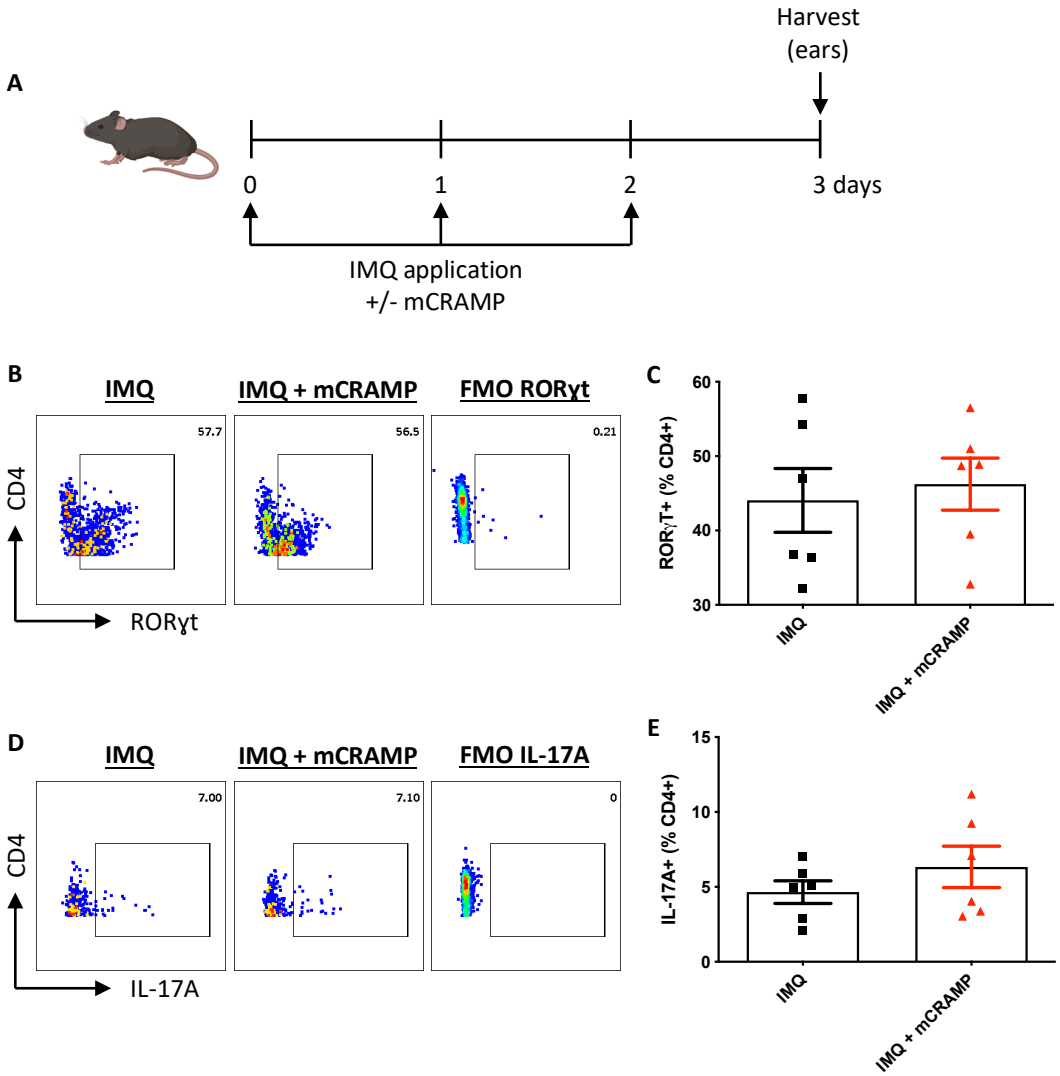


Figure 5.2: mCRAMP at the site of inflammation does not boost IL-17 production. (A) 5% imiquimod (IMQ) was applied daily to WT ears for 3 days, with or without synthetic mCRAMP (10 µg/mouse). Ears were digested on day 3 for analysis **(B)** Representative plots of $CD4^+$ $ROR\gamma t^+$ T cells in the ears on day 3 **(C)** Percentages of $CD4^+$ $ROR\gamma t^+$ T cells in the ears on day 3 **(D)** Representative plots of $CD4^+$ $IL\text{-}17A^+$ T cells in the ears on day 3 **(E)** Percentages of $CD4^+$ $IL\text{-}17A^+$ T cells in the ears on day 3. Data shown is mean +/- standard error. N = 6. IMQ: imiquimod.

5.3.3 mCRAMP can be found in the lymph nodes of mice inoculated with HKST

The data presented above suggests that T cells sense mCRAMP in the lymph nodes during inflammation *in vivo*. To confirm the presence of this host defence peptide in lymph nodes during inflammation, WT mice were inoculated with HKST in the top of each hind paw (25 μg/paw, subcutaneous) and the draining popliteal lymph nodes harvested and stained for mCRAMP (**Figure 5.3 A**; experiment performed by Virginia Alessandrini). As predicted, no mCRAMP was observed in sections from naïve mice. However, mCRAMP was detected in the lymph nodes following HKST inoculation, as early as day 1 and as late as day 7. mCRAMP-expressing cells located to the sub-capsular sinus (SCS) on day 1, where leukocytes have been shown to enter the lymph node via the afferent lymphatics[506]. These became more dispersed with time and were scattered throughout the organ by day 7.

5.3.4 mCRAMP is released from neutrophils in the lymph nodes of mice following inoculation with HKST

I hypothesised that neutrophils are the cellular source of mCRAMP that is responsible for amplifying Th17 differentiation following inoculation with HKST. There are several steps throughout the T cell differentiation process that can potentially be influenced by neutrophils and therefore mCRAMP. For instance, neutrophils can be found in the circulation, as well as in the lymph nodes and inflamed tissues[155].

Neutrophils are the primary producers of mCRAMP but other cell types, such as activated monocytes and macrophages, have been shown to express the peptide[277]. To confirm that mCRAMP$^+$ cells were indeed neutrophils and that positive staining was not simply a result of other cells producing or internalising it, additional sections of popliteal lymph nodes harvested from mice following inoculation with HKST were stained with DAPI, mCRAMP and the neutrophil-specific marker Ly6G (**Figure 5.3 B**; experiment performed by Katie Smith)[507]. Co-localisation of all three stains confirmed that mCRAMP-expressing neutrophils can be found in the lymph nodes of mice during inflammation induced by HKST: 85% of mCRAMP$^+$ cells were Ly6G$^+$ on day 7 (analysis by Katie Smith). Interestingly, extracellular mCRAMP was also observed, which could be a result of neutrophil degranulation or NETosis.

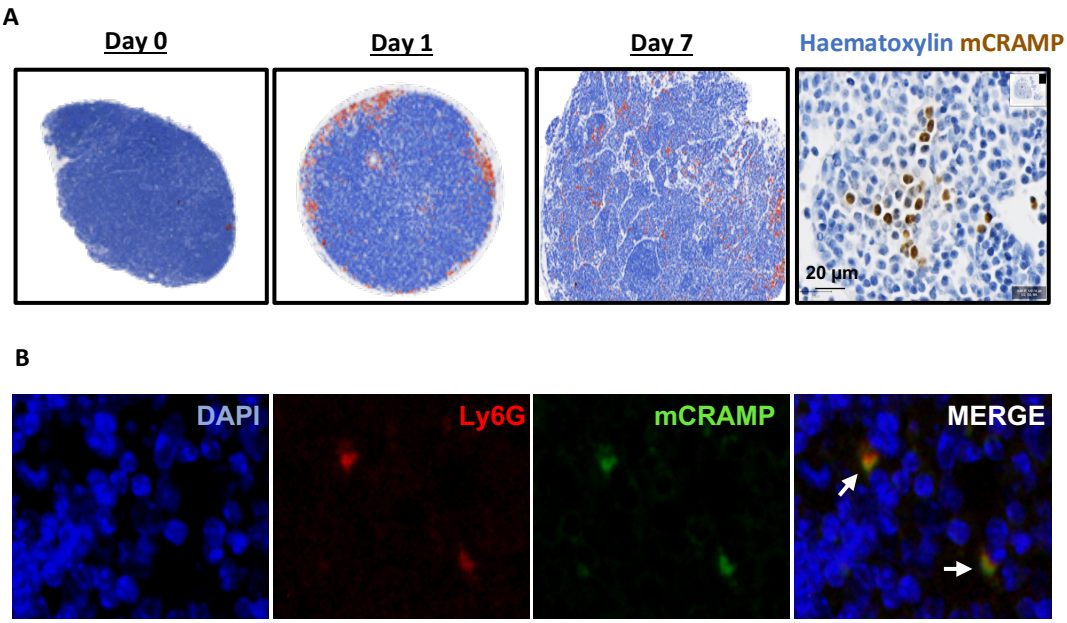


Figure 5.3: Neutrophil-derived mCRAMP can be detected in the lymph nodes following inoculation with HKST. WT mice were inoculated with 25 µg HKST in the top of each hind paw **(A)** Draining popliteal lymph nodes were removed on days 0, 1 and 7 and stained for mCRAMP. mCRAMP-expressing cells were detected as DAB^+ (staining performed by Virginia Alessandrini) **(B)** Draining popliteal lymph nodes were removed on day 1 and stained for DAPI, Ly6G and mCRAMP (staining performed by Katie Smith).

5.3.5 mCRAMP-deficient neutrophils downregulate RORγt and AHR expression in CD4⁺ T cells cultured under Th17-driving conditions

I have provided evidence, in collaboration with others, that suggests neutrophils are the cellular source of mCRAMP within the lymph nodes and that this early mCRAMP - T cell interaction enhances Th17 differentiation during inflammation induced by HKST. I therefore next investigated whether or not neutrophils are capable of increasing the development of IL-17-producing $CD4^+$ T lymphocytes *in vitro*, and if so, whether this is mCRAMP-dependent (**Figure 5.4**).

Neutrophils were isolated from the bone marrow of WT and mCRAMP KO mice by EasySep and activated with cytochalasin B and fMLF for 30 minutes to induce degranulation, as shown by Sato and colleagues[508]. Whole single cell splenic suspensions were then cultured with these bone marrow-isolated neutrophils (at a ratio of 1:1) under Th17-driving conditions for 2 days, as previously described.

Figure 5.4 A shows that WT neutrophils increased the percentage of $CD4^+$ IL-$17A^+$ T cells from 15.57% (+/- 1.75) to 20.40% (+/- 2.02), although this was not statistically significant (P = 0.08). In comparison, this rose to 25.13% (+/- 1.75) in response to synthetic mCRAMP and only 18.73% (+/- 2.54) following culture with mCRAMP-deficient neutrophils. The moderate increase in the frequency of IL-17-producing $CD4^+$ T cells induced by WT neutrophils could be a result of a lower concentration of biologically active mCRAMP in neutrophil preparations. Indeed, I have previously shown that the increase in $CD4^+$ IL-$17A^+$ T cells induced by mCRAMP is concentration-dependent (**Chapter 3: Figure 3.8 H**). Nonetheless, these results hint that neutrophils might increase Th17 polarization *in vitro*, although it is not possible to conclude whether this is mCRAMP-dependent.

While mCRAMP decreased the proportion of $CD4^+$ IFNγ^+ T cells as expected (from 4.65% +/- 1.02 to 2.22% +/- 0.41), WT neutrophils did not. Conversely, mCRAMP KO neutrophils significantly increased the frequency of IFNγ-producing $CD4^+$ T lymphocytes (12.35% +/- 4.17) (**Figure 5.4 B**). However, the results were highly variable between experiments and should therefore be analysed with caution. Nonetheless, I have shown that mCRAMP skews T helper cell differentiation away from the Th1 lineage. An increase in the percentage of $CD4^+$ IFNγ^+ T

lymphocytes in Th17 cultures containing mCRAMP KO neutrophils could therefore reflect increased Th1 polarisation, which arises a result of mCRAMP-deficiency and therefore a lack of suppression of IL-2 and T-bet.

Despite increasing the frequency of $CD4^+$ IL-$17A^+$ T cells, WT neutrophils had no effect on the expression of RORγt (**Figure 5.4 C**) or AHR (**Figure 5.4 D**). On the other hand, mCRAMP-deficient neutrophils significantly decreased the percentage of $CD4^+$ RORγt^+ T cells from 71.25% (+/- 1.79) to 61.73% (+/- 3.05), as well as the proportion of $CD4^+$ AHR^+ T lymphocytes from 49.14% (+/- 4.15) to 22.65% (+/- 4.38).

Taken together, these results imply that neutrophils from WT mice might be capable of enhancing Th17 differentiation *in vitro*. Conversely, those from mCRAMP KO mice appeared to suppress both RORγt and AHR expression, which could be indicative of increased Th1 polarization. However, further investigation is required to confirm these results.

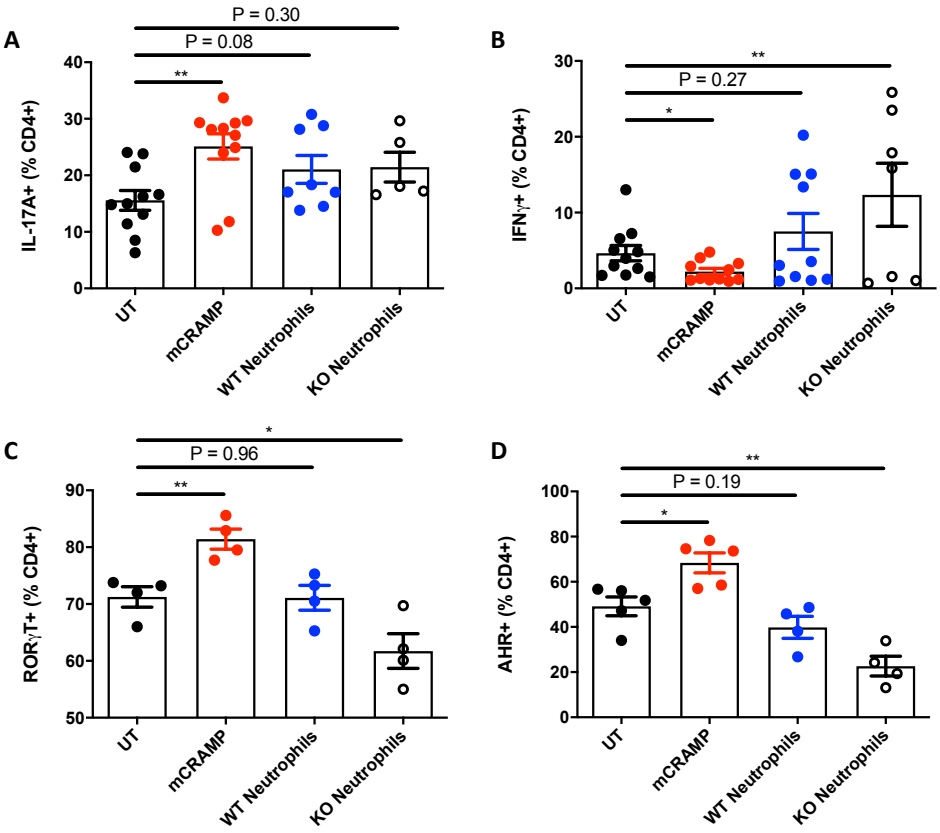


Figure 5.4: WT neutrophils have no significant effect on Th17 differentiation, whereas mCRAMP-deficient neutrophils decrease RORγt and AHR expression. Whole single cell splenic suspensions were cultured under Th17-driving conditions with 2.5 μM synthetic mCRAMP, WT neutrophils (1:1) or mCRAMP-deficient neutrophils (KO; 1:1) **(A)** Percentages of $CD4^+$ $IL\text{-}17A^+$ T cells on day 2 **(B)** Percentages of $CD4^+$ $IFN\gamma^+$ T cells on day 2 **(C)** Percentages of $CD4^+$ $ROR\gamma t^+$ T cells on day 2 **(D)** Percentages of $CD4^+$ AHR^+ T cells on day 2. Data shown is mean +/- standard error. Graphs represent multiple experiments. Statistical significance (where * represents < 0.05 and ** < 0.01) was determined using an unpaired t-test. UT: untreated; WT: wildtype; KO: knockout.

5.3.6 Neutrophil depletion enhances Th17 responses in vivo following inoculation with heat-killed S. typhimurium

The results discussed above suggest that neutrophils are potentially capable of boosting Th17 differentiation *in vitro*. To investigate the importance of neutrophils in amplifying Th17 responses *in vivo*, I repeated the HKST model in WT and neutrophil-depleted mice (**Figure 5.5**).

WT C57BL6/J mice were injected intraperitoneally with an anti-Ly6G antibody (αRIMP1) to deplete neutrophils on days -1, 1 and 3. All mice were inoculated with 25 μg HKST in the top of each paw on day 0 and the draining popliteal lymph nodes harvested on day 7 for phenotyping. Based on previous data, I hypothesized that neutrophil depletion would impair the development of IL-17-producing $CD4^+$ T lymphocytes and result in a similar phenotype to that observed in mCRAMP KO mice.

Neutrophil depletion was assessed by confirming the absence of SSC-A^{hi} $CD11b^+$ cells within the spleen (**Figure 5.5 D**). Neutrophil-depleted mice displayed an enlarged spleen (**Figure 5.5 C**) and flow cytometric analysis revealed an influx of SSC-A^{lo} $CD11b^+$ cells on day 7 (**Figure 5.5 B**). Bruhn et al. determined that neutrophil depletion is mediated predominantly by macrophages[509]. These SSC-A^{lo} $CD11b^+$ cells therefore likely represented this innate leukocyte.

Surprisingly, whilst the percentage of $CD4^+$ $CD44^+$ T cells was not significantly different between neutrophil-depleted mice and controls, the geometric mean of PD1 expression rose significantly from 296 +/- 11 to 396 +/- 21 (**Figure 5.5 F & G**). This suggests that $CD4^+$ T cells are more activated in neutrophil-depleted mice inoculated with HKST.

Furthermore, neutrophil depletion led to a significant rise, from 2.16% (+/- 0.07) to 3.41% (+/- 0.23), in the percentage of $CD4^+$ IL-$17A^+$ T cells within the lymph nodes (**Figure 5.5 H**). The concentration of IL-17A in cell culture supernatants following re-stimulation of single cell lymph node suspensions was assessed by ELISA (**Figure 5.5 I**). Cells from neutrophil-depleted mice re-stimulated for 3 days with αCD3 (2.5 μg/mL) produced more IL-17A than those from control animals (20.16 compared to 5.96 ng/mL). In addition, re-stimulation with HKST (1

μg/mL) also led to an increase in IL-17A production by cells from neutrophil-depleted mice (from 2.05 to 6.82 ng/mL).

These results were contrary to what was expected. One possible explanation is that neutrophil depletion resulted in some antibody-mediated cytotoxicity, which could have led to the unprogrammed cell death of neutrophils and a subsequent release/burst of mCRAMP. In addition, Patel et al. recently demonstrated that systemic depletion of neutrophils results in a dysregulated negative feedback IL-23 – IL-17 – G-CSF regulatory axis in the periphery[510]. Increased levels of IL-23 could therefore provide an explanation as to why neutrophil depletion led to an apparent increase in the percentage of $CD4^+$ $IL\text{-}17A^+$ T cells following inoculation with HKST.

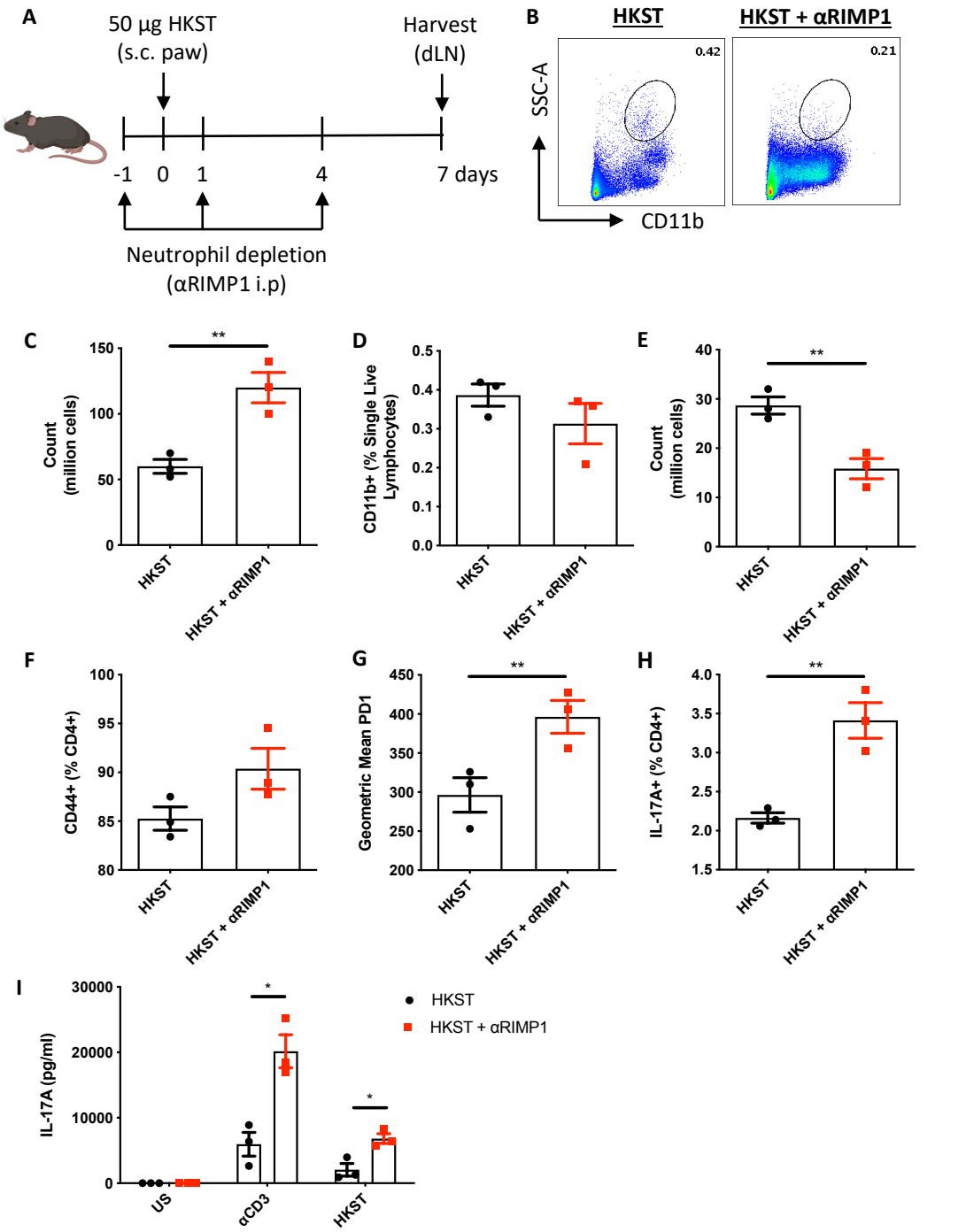


Figure 5.5: Neutrophil depletion enhances Th17 responses following inoculation with heat-killed *S. typhimurium*. (A) WT and neutrophil-depleted mice were inoculated with 25 µg HKST in to the top of each hind paw and the draining popliteal lymph nodes removed 7 days later for analysis **(B)** Representative plots of CD11b^{+} cells in the spleen on day 7 **(C)** Total number of cells isolated from the spleen on day 7 **(D)** Percentages of SSC-A^{hi} CD11b^{+} cells in the spleen on day 7 **(E)** Total number of cells isolated from the popliteal lymph nodes **(F)** Percentages of CD4^{+} CD44^{+} T cells in the lymph node on day 7 **(G)** Geometric Mean of PD1 expression by CD4^{+} T cells in the lymph node day 7 **(H)** Percentages of CD4^{+} IL-17A^{+} T cells in the lymph node on day 7 **(I)** Concentration of IL-17A in cell culture supernatants following 3 days of re-stimulation with αCD3 (5 µg/mL) or HKST (1 µg/mL). Data shown is mean +/- standard error. N = 3. Statistical significance (where * represents < 0.05 and ** < 0.01) was determined using an unpaired t-test. HKST: heat-killed *Salmonella typhimurium;* dLN: draining lymph node; s.c.: subcutaneous; i.p: intraperitoneal; US: unstimulated

5.4 Discussion

mCRAMP-deficient mice cannot produce IL-17 following inoculation with HKST and I have shown in previous chapters that mCRAMP enhances Th17 differentiation *in vitro*. I therefore sought to identify where T cells sense this host defence peptide *in vivo* during inflammation, as well as its cellular source.

5.4.1 T cells sense mCRAMP in the lymph nodes during inflammation

$CD4^+$ T lymphocytes cultured under Th17-driving conditions required mCRAMP during the first 24 hours in order to boost Th17 differentiation *in vitro*: the addition of mCRAMP on day 1 had no effect on the percentage of $CD4^+$ $IL\text{-}17A^+$ $IL\text{-}17F^-$, $IL\text{-}17A^-$ $IL\text{-}17F^+$ or $IL\text{-}17A^+$ $IL\text{-}17F^+$ T cells.

Miller and colleagues used two-photon microscopy to image single cell dynamics of naïve $CD4^+$ T cells and *in vivo*-labelled DCs in lymph nodes following immunization with OVA[499]. They demonstrated that T cell interactions with antigen-presenting DCs could be categorized into several stages that overlap[499,511]. Within the first 6 hours, transient, serial encounters led to the upregulation of T cell activation markers: by 2 hours, around 40% of antigen-specific T cells had up-regulated CD69[499]. T cells then entered an extended period lasting 12-14 hours in which T cell clusters around DCs could be observed[499]. These were characterized by more stable binding events that lasted for hours[499]. By 24 hours, 80% of antigen-specific T lymphocytes were $CD69^+$ and highly motile[499]. Based on these observations, exposure to mCRAMP from day 0, when the T cells are naïve and un-activated, models them sensing the peptide in the lymph node. Conversely, adding the peptide a day later, when the cells have already received activation and differentiation signals, models them coming into contact with mCRAMP in the inflamed tissue. My results therefore suggest that mCRAMP acts specifically on naïve $CD4^+$ T cells, as opposed to fully differentiated or committed lymphocytes. Furthermore, the data implies that during inflammation induced by HKST, T lymphocytes sense mCRAMP in the lymph nodes, which boosts Th17 differentiation.

This was supported by an experiment in which I applied cream containing 5% imiquimod (IMQ), spiked with synthetic mCRAMP, daily to the ears of WT mice. This particular model of

acute skin inflammation is the most widely used mouse model for preclinical studies of psoriasis[501]. Imiquimod is an immune response modifier that activates the NF-κB pathway[512]. This single, synthetic innate antigen receptor ligand induces localized skin and systemic inflammation, primarily through the activation of TLR7/8, which is expressed by monocytes, macrophages and pDCs[500,501]. This leads to the migration of Langerhans cells from the treated skin into the draining lymph nodes where they activate T lymphocytes and the adaptive immune response[502,503]. Other features of IMQ-induced skin inflammation include the development of psoriasis-like histological features (epidermal hyperplasia), activation of pro-inflammatory signalling pathways and the subsequent recruitment of cellular infiltrates ($CD4^+$ T cells, neutrophils, $CD11c^+$ DCs and pDCs)[500–502]. $CD4^+$ T cells and the IL-23/IL-17 axis play a pivotal role in mediating the response to imiquimod, demonstrated by the fact that IMQ-induced dermatitis is partially dependent on the presence of T cells and is completely blocked in mice deficient for IL-23 or IL-17R[500]. Indeed, Ueyama et al. demonstrated that IMQ induces the production of cytokines required for Th17 differentiation by mouse pDCs via TLR7[504].

In order to evaluate the early inflammatory process, mice were culled and the ears harvested on day 3, before significant increases in ear thickness were observed (this typically occurs from days 5-6 onwards). In this model, I hypothesised that T cells encounter the synthetic mCRAMP following activation, differentiation and recruitment to the inflamed tissue. Quantification of IL-17-expressing cells in the ears on day 3 revealed that the synthetic peptide had no effect on the frequencies of $CD4^+$ $ROR\gamma t^+$ or $IL\text{-}17A^+$ T cells. This supports the hypothesis that T lymphocytes must sense mCRAMP in the lymph nodes during the first 24 hours of activation in order to enhance Th17 differentiation.

In a separate experiment performed by Emily Gwyer Findlay, the HKST inoculum was spiked with 10 μg synthetic mCRAMP, which led to an increase in the frequency of IL-17-producing $CD4^+$ T lymphocytes within the draining popliteal lymph nodes **(Figure 5.6**; Emily Gwyer Findlay). How the synthetic peptide is transported to this lymphoid organ where it can act on naïve $CD4^+$ T lymphocytes is unknown. Xhindoli et al. suggested that cathelicidin is 'sticky' due to the fact that it favours interactions with any molecular surface offering appropriate hydrophobic, electrostatic and H-bonding properties[314]. In addition, several other immune

cells, such as macrophages, have been shown to internalise cathelicidin in a P2X7R-dependent manner, which could then carry the peptide elsewhere[513]. However, it is important to note that the effects of mCRAMP on Th17 differentiation and IL-17 production are potentially mediated indirectly. For example, as discussed previously, the possibility that mCRAMP influences the differentiation of DCs that subsequently enhance the development of IL-17-producing T lymphocytes cannot be excluded (**Chapter 3: section 3.4.2.1**).

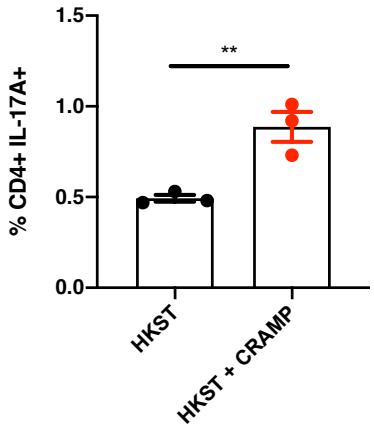


Figure 5.6: Synthetic mCRAMP boosts IL-17 production by lymph node CD4⁺ T cells following inoculation with HKST. WT mice were inoculated with 12.5 µg HKST in the top of each hind paw, with or without 10 µg synthetic mCRAMP. The draining popliteal lymph nodes were removed on 7 for phenotyping by flow cytometry. Graph shows percentages of CD4⁺ IL-17A⁺ T cells (experiment performed by Emily Gwyer Findlay)

5.4.2 Neutrophils are the cellular source of mCRAMP in the lymph nodes during inflammation induced by HKST

My results suggest that T cells sense mCRAMP in the lymph nodes following inoculation with HKST, which amplifies Th17 responses. Neutrophils are one of the prominent producers of mCRAMP and have the capacity to migrate to the lymph nodes in response to different stimuli[285,514]. For example, neutrophils can shuttle live mycobacterium bacilli to lymphoid tissues following intradermal BCG vaccination[206]. Co-localised staining for Ly6G and mCRAMP in the lymph nodes of HKST-inoculated mice indicated that neutrophils may be the cellular source of this host defence peptide in this particular model of inflammation.

Neutrophils can enter the lymph node by exiting the circulation through high endothelial venules (HEVs)[515]. For instance, HEVs are the major route of entry of blood-borne neutrophils into tumour-draining lymph nodes[167]. This is dependent on interactions between neutrophil L-selectin and CXCR2 with addressin and CXCL2, expressed by HEVs[167]. Beauvillain et al. also found that the recruitment of neutrophils to lymph nodes via this pathway requires CCR7: injection of complete Freund's adjuvant (CFA) failed to induce the rapid recruitment of neutrophils to the lymph nodes in CCR7-deficient mice[169]. On the other hand, neutrophils can also be recruited to lymph nodes via the afferent lymphatics, in a process mediated by CD11b and CXCR4[168]. Following inoculation with HKST, mCRAMP localised to the outer rim of the lymph node on day 1. This area, known as the sub-capsular sinus (SCS), represents where neutrophils enter the lymph node via the afferent lymphatics, suggesting that this is the route of entry in this model of inflammation[168].

Antigen-bearing DCs and T lymphocytes have also been shown to traffic via the afferent lymphatics and enter the lymph nodes through the SCS[11,516]. For instance, Rantakari et al. demonstrated that T cells could be detected within the lymph node parenchyma in close proximity to the SCS as early as four hours after adoptive transfer[11,517]. One might therefore speculate that this is where naïve T lymphocytes encounter mCRAMP$^+$ cells for the first time. Moreover, our staining showed that with time, mCRAMP$^+$ neutrophils became more scattered and dispersed throughout the lymph node following inoculation with HKST: by day 7, they appeared to localize to the deeper paracortex, which is populated predominantly by T lymphocytes[518]. It is within this T cell zone where naïve T lymphocytes receive signals that trigger their activation and differentiation[518]. mCRAMP$^+$ neutrophils were consequently in close proximity to T cells as early as day 1 following inoculation, as well as up to 7 days later, and therefore had the potential to induce changes that enhance Th17 differentiation.

Initially, there was some debate as to whether lymph node neutrophils were simply in transit or involved in lasting interactions with other immune cells that could subsequently modulate the development of the adaptive immune response. Maletto et al. demonstrated that immune complexes generated by injecting OVA into the footpad of OVA/CFA-immunized mice induced the migration of OVA$^+$ neutrophils to the draining lymph nodes[205]. Subsequently, it was found that this influx of OVA$^+$ neutrophils led to an increase in the

proliferation, activation and cytokine production of $CD4^+$ T cells[519]. In addition, Hampton and colleagues found that during skin inflammation, neutrophils migrated within lymphatic vessels and re-localised to the SCS of draining lymph nodes in response to microbial infection[168]. This resulted in increased lymphocyte proliferation and the enhancement of adaptive immunity[168].

The studies discussed above indicate that lymph node neutrophils can have profound effects on the development of adaptive immune responses. Neutrophils possess a huge repertoire of intracellular mediators that are released upon degranulation and during NETosis or necrosis, including mCRAMP[229]. However, whether or not neutrophils undergo any of these processes within the lymph node remains unclear. Odobasic et al. demonstrated that neutrophils deposited myeloperoxidase (MPO) in the lymph nodes 4 hours after OVA/LPS injection, and that this enzyme inhibited antigen uptake and processing by DCs[269]. Furthermore, Krishnamoorthy and colleagues were able to identify neutrophil cytoplasts in the mediastinal lymph nodes of mice that had been exposed to an aeroallergen and endotoxin during sensitization in a model of allergic asthma[233]. Neutrophil cytoplasts are defined as the remnants of NETs following the expulsion of their DNA[233]. These enucleated cell bodies were capable of activating lung DCs *in vitro* to trigger antigen-specific IL-17 production from naïve $CD4^+$ T cells[233]. In addition, Ahn et al. explored the involvement of NETs in adult-onset Still disease by performing immunohistochemical analysis of skin and lymph node biopsies[520]. They identified neutrophil elastase- and MPO-positive inflammatory cells in the lymph nodes of patients, which were expressed in fibre form[520]. The presence of extracellular mCRAMP in the lymph nodes of mice inoculated with HKST suggests that neutrophils can undergo these processes that lead to the release of inflammatory mediators.

Activated neutrophils have been shown to produce TGFβ[195,242]. For instance, Kamenyeva and colleagues demonstrated that the mobilization of bone marrow neutrophils to the draining lymph nodes following challenge with *S. aureus* led to the suppression of the early humoral response and that this was TGFβ-dependent[195]. Moreover, highly pure human neutrophils activated via TLR8 produce IL-23 and can subsequently promote Th17 polarization[521]. Both TGFβ and IL-23 play important roles in Th17 development[26]. Furthermore, I have shown that mCRAMP boosts the generation of Th17 cells in a TGFβ-dependent manner. One might

therefore speculate that neutrophils play a dual role in driving Th17 differentiation by simultaneously releasing mCRAMP and producing Th17-stimualting cytokines such as TGFβ and IL-23.

5.4.3 Neutrophils potentially promote Th17 differentiation in vitro

I have shown that neutrophils are a potential source of mCRAMP within the lymph nodes that boost Th17 responses following inoculation with HKST. I therefore sought to determine whether neutrophils promoted Th17 differentiation *in vitro*, similarly to mCRAMP. Neutrophils from wildtype mice had no significant effect on IL-17A/IFNγ production or RORγt/AHR expression. However, there was a trend indicating that culturing whole single cell splenic suspensions with WT neutrophils increased the percentage of $CD4^+$ $IL\text{-}17A^+$ T cells.

The concentration of mCRAMP in neutrophil preparations was not determined and it is difficult to predict how much is released following activation and degranulation. Sørensen et al. determined that the concentration of hCAP-18 in human neutrophils is approximately 0.627 μg/10^6 cells. Assuming mouse neutrophils are similar and that all neutrophils degranulated, I therefore predict that there was around 1.3 μM in my neutrophil suspensions. This is only half the amount of synthetic mCRAMP that I typically added to Th17 cultures (2.5 μM).

Moreover, hCAP-18 refers to the full length pre-peptide: cathelicidin requires proteolytic processing by proteinase-3 in order to release the biologically active fragment[289]. Proteinase-3 is stored in primary, azurophilic granules, which prevents the unwanted intracellular processing of cathelicidin in resting neutrophils[281]. Neutrophil degranulation occurs in the reverse but ordered sequence of synthesis: secretory granules require minimal cellular stimulation for release, whereas azurophilic granules require a very powerful agonist[159]. In the present study, neutrophils were primed with cytochalasin B and stimulated with fMLF (N-formyl-Met-Leu-Phe). fMLF is a potent inducer of neutrophil degranulation and has been shown to stimulate the extracellular release of azurophilic granule contents[508,522]. Stimulation with fMLF therefore ensured the release of proteinase-3 and that the pre-pro-peptide could be cleaved. However, it is also possible that not enough of the enzyme was

released or that these neutrophil preparations contained too many immature PMN that had not yet fully undergone neutrophil granule formation. Future work will involve confirming neutrophil degranulation and the presence of biologically active mCRAMP. For example, a western blot could be performed on neutrophil supernatants, alongside samples of known concentration (e.g. synthetic CRAMP) to qualitatively assess how much is present.

Previous Th17 cultures were treated with 2.5 μM synthetic mCRAMP, which is physiological in scenarios of inflammation where there is an influx of neutrophils. In the present study, neutrophils were added at a ratio of approximately 1:1. It is therefore likely that the amount of mCRAMP released did not equate to 2.5 μM. Future experiments will therefore include increasing the ratio of neutrophils to splenocytes (e.g. 5:1), which should be more physiologically relevant. In addition, purified $CD4^+$ T lymphocytes will be used instead of whole single cell splenic suspensions, as it is possible that other cell types within the spleen could interfere.

Neutrophils from mCRAMP-deficient mice did not significantly increase the frequency of IL-17-producing $CD4^+$ T lymphocytes, although the percentage of $CD4^+$ IL-$17A^+$ T cells was similar to that obtained following culture with WT neutrophils. However, a significant increase in the percentage of $CD4^+$ IFNγ^+ T cells was observed compared to untreated samples. In addition, KO neutrophils significantly decreased the frequency of AHR- and RORγt-expressing $CD4^+$ T lymphocytes. One possible explanation for this is that a currently unspecified neutrophil mediator promoted Th1 polarization due to the absence of mCRAMP, which normally counteracts its action in the presence of Th17-stimulating cytokines. For example, lactoferrin enhances Th1 differentiation in humans: Hwang et al. demonstrated that the addition of lactoferrin to the BCG vaccine boosted the generation of BCG-specific Th1 responses and increased IFNγ production[523]. To corroborate this, future experiments will involve examining the expression of other Th1 markers (e.g. T-bet) in T cell – neutrophil co-cultures.

It is important to note that there was a high degree of variability between experiments, particularly with regards to IFNγ production. This could be an indication that several of the mice used were sick or infected with an unknown pathogen. Furthermore, the mCRAMP KO

mice used throughout the majority of this study were re-derived into a separate animal facility, which is considered 'cleaner'. Differences in the microbiome of these mice could have profound effects on T helper cell differentiation and cytokine production[524]. For example, Ivanov and colleagues found that C57BL/6 mice obtained from different commercial vendors displayed significant differences in the proportion of Th17 cells in the gastrointestinal lamina propria[90]. They went on to demonstrate that colonization of the small intestine with segmented filamentous bacteria (SFB) induces Th17 development and that mice that have few Th17 cells lack this particular commensal microbe[91].

Nonetheless, taken together, these results suggest that neutrophils potentially promote Th17 differentiation *in vitro,* although it was not possible to conclude whether this is mCRAMP-dependent or not. Further investigation is therefore required.

5.4.4 Neutrophil depletion does not impair the development of the Th17 response following inoculation with HKST

To further examine the effects of neutrophils on the development of Th17 responses, I inoculated wildtype and neutrophil-depleted mice with HKST. I hypothesized that if neutrophil-derived mCRAMP is important for boosting Th17 differentiation, neutrophil-depleted animals would display a similar phenotype to mCRAMP KO mice and fail to produce IL-17. However, the percentage of $CD4^+$ $IL\text{-}17A^+$ T cells within the draining popliteal lymph nodes was significantly increased in neutrophil depleted mice compared to the control group. Furthermore, cells from neutrophil-depleted mice produced significantly more IL-17A following re-stimulation with both αCD3 and HKST.

There are several possible explanations for this. Firstly, the intraperitoneal injection of an anti-Ly6G antibody depletes neutrophils by targeting them for clearance by macrophages; Bruhn et al. found that anti-Ly6G-mediated neutrophil depletion is abrogated in the absence of this cell type[509]. Indeed, I observed a significant influx of $SSC\text{-}A^{lo}$ $CD11b^+$ cells in the spleen following treatment. However, antibody-dependent cellular cytotoxicity (ADCC) has been suggested to also play a role[525]. ADCC refers to the immune mechanism through which Fc receptor-bearing effector cells recognize and kill antibody-coated targets[526]. One might therefore speculate that the lysis of a large number of neutrophils could lead to a sudden

release/burst of mCRAMP, which could in turn get taken up by other immune cells and transported to the lymph nodes where it enhances Th17 differentiation.

On the other hand, Patel et al. recently demonstrated that chronic, systemic depletion of neutrophils results in the failure of apoptotic neutrophils to trigger the negative feedback IL-23 – IL-17 – G-CSF regulatory axis in the periphery[510]. More specifically, during homeostasis, phagocytosis of transmigrated, apoptotic neutrophils by resident macrophages and DCs results in the suppression of their intrinsic IL-23 production[510,527]. IL-23 is important for the maintenance, stabilisation and pathogenicity of Th17 cells and is therefore a potent regulator of IL-17 expression[510]. An increase in the percentage of $CD4^+$ $IL\text{-}17A^+$ T cells could therefore be a result of sustained production of IL-23 from macrophages and DCs, whose inhibition normally prevents uncontrolled Th17 responses and the excessive production of harmful IL-17.

In order to overcome these issues and address the question as to whether neutrophil-derived mCRAMP is truly required for the development of a Th17 response following inoculation with HKST, it would be helpful to generate a mouse line that is deficient for mCRAMP specifically in neutrophils. However, efforts by the group thus far have proven unsuccessful. This is possibly due to the fact that mCRAMP is not synthesised by neutrophils *de novo* upon stimulation[286]. Instead, it is produced at the myelocyte and metamyelocyte stage of neutrophil maturation and stored as an inactive pre-pro-peptide in secondary granules[286]. It is therefore difficult to target due to how early it is expressed within the developing neutrophil (earlier than targets for the cre-lox system such as MPR8).

5.5 Summary

To summarize, I have provided evidence that suggests naïve $CD4^+$ T cells sense mCRAMP in the lymph nodes during inflammation, which enhances Th17 responses *in vivo*. Immunofluorescent staining of lymph nodes taken from mice following HKST inoculation demonstrated shown that mCRAMP co-localised with Ly6G, indicating that neutrophils may be the cellular source.

However, culturing whole single cell splenic suspensions with WT neutrophils *in vitro* did not significantly increase RORγt or IL-17 expression, or at least not to the same extent as synthetic mCRAMP. On the other hand, mCRAMP-deficient neutrophils significantly increased IFNγ production and downregulated RORγt expression compared to UT controls. This potentially reflects an increase in the development of Th1 lymphocytes as a result of the absence of mCRAMP, which normally skews T helper cell polarization away from this lineage.

Further investigation is required in order to validate the role of neutrophil-derived mCRAMP in the development of Th17 responses during inflammation. A mouse line that is specifically deficient for neutrophil-mCRAMP would be highly beneficial but this has proven technically challenging thus far.

CHAPTER 6

mCRAMP is a Tc17 Differentiation Enhancing Factor

6.1 Introduction

$CD8^+$ T lymphocytes can be subdivided into different subsets, reminiscent of T helper cell classification[528]. Tc1 cells are considered canonical cytotoxic T lymphocytes (CTLs) that are capable of efficiently killing infected or malignant cells bearing their target antigen by releasing cytotoxic molecules into the immunological synapse[528]. Eomes and T-bet drive the CTL differentiation program by promoting the expression of IFNγ and the cytolytic molecule granzyme B[529]. On the other hand, Tc17s express type 17 markers, such as RORγt, IL-23R and IL-17, display highly suppressed cytotoxic activity (diminished levels of IFNγ and granzyme B) and therefore lack lytic function *in vitro*[530–532].

A lot of information regarding the differentiation of Tc17 cells stems from mouse studies: naïve $CD8^+$ T cells activated in the presence of IL-6 and TGFβ develop into IL-17-producing Tc17 cells, similarly to $CD4^+$ T lymphocytes[531,533]. However, the exact conditions required for human Tc17 polarisation are still unclear[533]. A limited number of studies have been published that show only very low frequencies of $CD8^+$ $IL\text{-}17^+$ T cells or low levels of IL-17 production following induction[533,534]. For example, Kondo and colleagues demonstrated that human naïve $CD8^+$ T cells cultured with TGFβ, IL-6, IL-1β, IL-23 and anti-IFNγ for 5 days, followed by the addition of IL-2 for another 4 days, induced the development of 0.11% $CD8^+$ $IL\text{-}17^+$ T cells[534]. It therefore remains to be seen whether human Tc17 cells require another inducing factor that is yet to be identified, or whether these low levels of polarization are simply due to technical challenges.

Compared to $CD4^+$ Th17 lymphocytes, Tc17s have received relatively little attention with regards to their role during inflammation. However, they have been detected in various chronic inflammatory disorders, suggesting that they contribute to immunopathology[533]. For example, Tc17 cells in psoriasis patients use CCR6 to home to a CCL20-enriched environment, where IL-17 synergistically acts with IFNγ to promote keratinocyte proliferation[535]. Furthermore, myelin oligodendrocyte glycoprotein (MOG)-specific $CD8^+$ T cells isolated from the lymph nodes and CNS of mice with EAE at the peak of disease express IL-17 *ex vivo*[531,533]. These cells do not express granzyme B, indicating that their potential pathogenicity is not

dependent on cytotoxic mechanisms and may rather be related to pro-inflammatory function[531,533].

Similarly to $CD4^+$ Th17 cells, the presence and activity of $CD8^+$ IL-17^+ T lymphocytes in autoimmune disease can be associated with levels of cathelicidin. For instance, LL-37 acting as an autoantigen has been suggested to be one of the causes of Tc17 activation in psoriatic skin[536]. Furthermore, elevated levels of LL-37 have been measured in the synovial fluid of patients with psoriatic arthritis, together with increased numbers of $CD8^+$ IL-17^+ T cells[537,538].

6.2 Aims

mCRAMP KO cannot produce IL-17 in response to inflammation induced by heat-killed *S. typhimurium* (HKST) and I have shown that mCRAMP enhances $CD4^+$ Th17 differentiation *in vitro*. I therefore sought to determine whether mCRAMP influences $CD8^+$ T cells in the same manner. The aims of this chapter were to examine the effects of synthetic mCRAMP on mouse Tc17 differentiation *in vitro*.

6.3 Results

6.3.1 $CD8^+$ T cells that develop in the absence of mCRAMP have normal cytokine responses

mCRAMP KO mice could not produce IL-17 in response to inoculation with HKST (**Chapter 1: Figure 1.10**). I have shown that $CD4^+$ T lymphocytes from naïve mCRAMP-deficient mice displayed similar cytokine responses to WT $CD4^+$ T cells (**Chapter 3: Figure 3.1**). However, IL-17-producing $CD8^+$ T lymphocytes can also contribute to a type-17 response. I therefore sought to determine whether $CD8^+$ T cells that developed in the absence of mCRAMP possessed an underlying defect that could contribute to the failure of IL-17 production in mCRAMP KO mice during inflammation induced by HKST.

I compared $CD8^+$ T lymphocyte cytokine production *ex vivo* in cells from the liver, lungs, spleen, mesenteric lymph nodes and Peyer's patches from naïve WT and mCRAMP KO mice by intracellular flow cytometry, in order to assess baseline cytokine responses (**Figure 6.1**).

There were no significant differences in the production of IL-17A, IL-17F, IFNγ, IL-22 or GM-CSF between the two genotypes in any of the tissues tested. However, there was a significant increase in the frequency of $CD8^+$ TNFα^+ T cells in the liver (45.48% +/- 3.59 compared to 32.45% +/- 3.87) and mesenteric lymph nodes (32.58% +/- 2.81 compared to 23.20% +/- 1.27) of mCRAMP KO mice. Despite this, these data suggest that like $CD4^+$ T lymphocytes, $CD8^+$ T cells that develop in the absence of mCRAMP do not have drastically different cytokine responses at resting state.

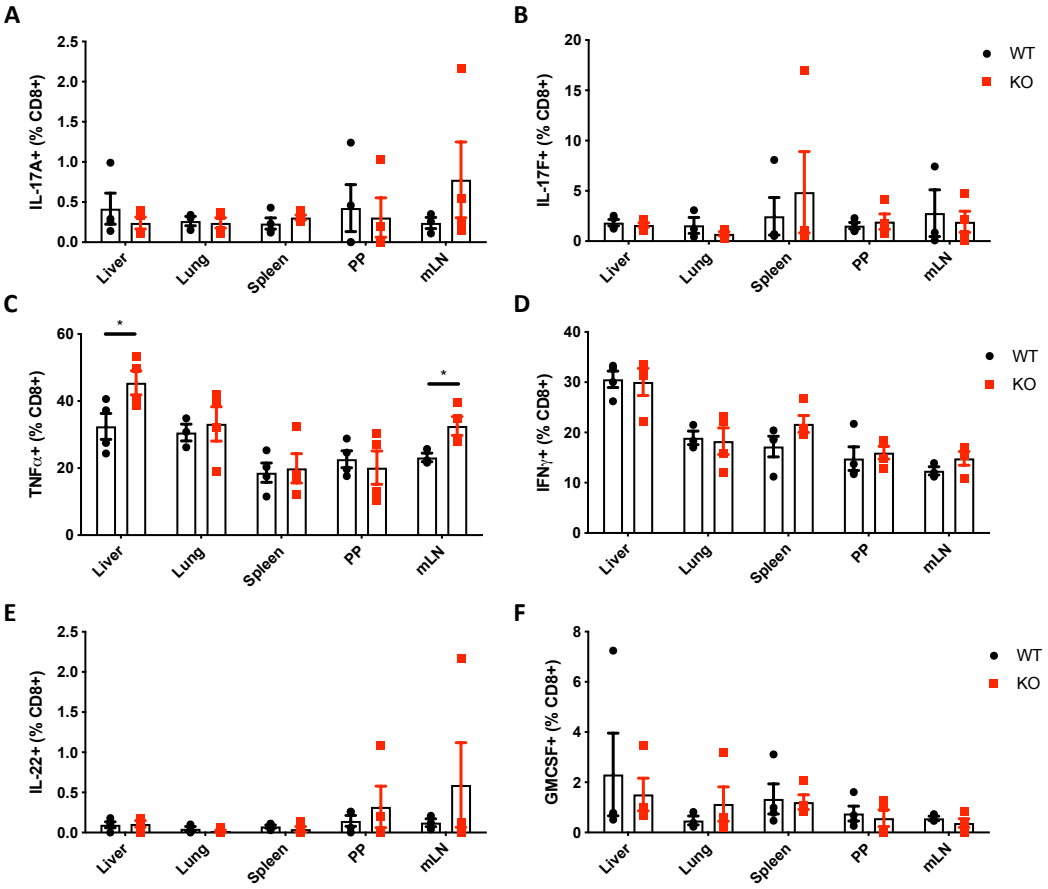


Figure 6.1: CD8⁺ T cells that develop in the absence of mCRAMP have relatively normal cytokine responses. CD8⁺ T cell cytokine production was assessed in the liver, lungs, mesenteric lymph nodes, Peyer's patches and spleen from naïve WT and mCRAMP KO mice **(A)** Percentages of CD8⁺ IL-17A⁺ T cells in WT and KO mice **(B)** Percentages of CD8⁺ IL-17F⁺ T cells in WT and KO mice **(C)** Percentages of CD8⁺ TNFα⁺ T cells in WT and KO mice **(D)** Percentages of CD8⁺ IFNɣ⁺ T cells in WT and KO mice **(E)** Percentages of CD8⁺ IL-22⁺ T cells in WT and KO mice **(F)** Percentages of CD8⁺ GM-CSF⁺ T cells in WT and KO mice. Data shown is mean +/- standard error. N = 4. Statistical significance (where * represents < 0.05) was determined using an unpaired t-test. WT: wild-type; KO: knockout.

6.3.2 CD8⁺ T cells from mCRAMP knockout mice are capable of producing IL-17 in response to exogenous cytokines

Next, I sought to determine whether CD8⁺ T cells from mCRAMP KO animals were capable of producing IL-17 *in vitro* in response to exogenous IL-17-inducing cytokines. Whole single cell splenic suspensions from WT and mCRAMP KO mice were cultured with IL-6, IL-23 and TGFβ for 2 days, using the same protocol as for the differentiation of CD4⁺ Th17 lymphocytes. IL-17A production was assessed by intracellular flow cytometry on day 2 (**Figure 6.2**).

There was no significant difference in the percentage of CD8⁺ IL-17A⁺ T cells between WT and KO mice. However, there was a trend suggesting that the frequency of IL-17-producing CD8⁺ T lymphocytes generated in Tc17 cultures was reduced when using cells from mCRAMP KO mice. These results indicate that CD8⁺ T lymphocytes from mCRAMP-deficient mice are capable of producing IL-17A *in vitro* in response to exogenous cytokines, although perhaps to a slightly lesser extent than WT cells.

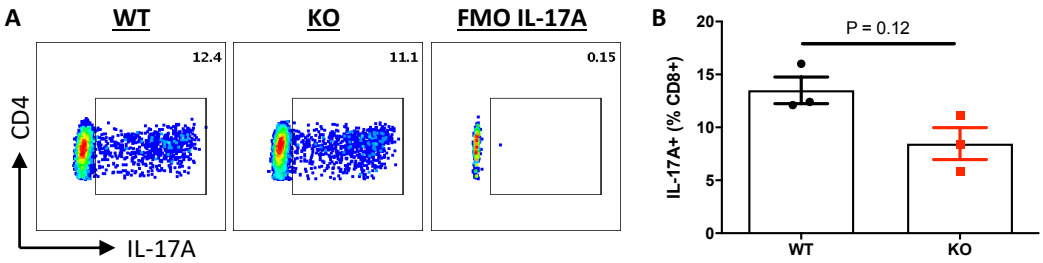


Figure 6.2: CD8⁺ T cells from mCRAMP knockout mice can produce IL-17A in response to exogenous cytokines. Whole single cell splenic suspensions from WT and mCRAMP KO mice were cultured under Tc17-driving conditions (20 ng/mL IL-6, 20 ng/mL IL-23, 3 ng/mL TGFβ) for 2 days **(A)** Representative plots of IL-17A production by CD8⁺ T cells from WT and mCRAMP KO mice on day 2, assessed by flow cytometry **(B)** Percentages of WT and mCRAMP KO CD8⁺ IL-17A⁺ T cells on day 2 following culture under Tc17-driving conditions. Data shown is mean +/- standard error. N = 3. A paired t-test was performed to determine statistical significance. WT: wild-type; KO: knockout.

6.3.3 mCRAMP increases CD8⁺ T cell activation but has no effect on IL-17 production following culture under non-lineage-driving conditions

$CD8^+$ T lymphocytes that developed in the absence of mCRAMP could produce IL-17 in response to exogenous cytokines and had relatively normal cytokine responses at resting state. I previously hypothesised that mCRAMP enhances the development of T cell immunity. To examine the effects of this peptide on $CD8^+$ T cells, I cultured whole single cell splenic suspensions under non-lineage-driving conditions with plate-bound αCD3, with or without synthetic mCRAMP.

Figure 6.3 shows that no significant differences in the total number of $CD8^+$ T lymphocytes were observed between untreated and mCRAMP-treated samples on days 1 and 2. However, mCRAMP increased the total number of $CD8^+$ T cells in non-polarising cultures on day 3, from 87,104 (+/- 18,290) to 110,723 (+/- 15,446). This is in contrast to observations made for $CD4^+$ T cells, which displayed no significant differences in total number following exposure to mCRAMP.

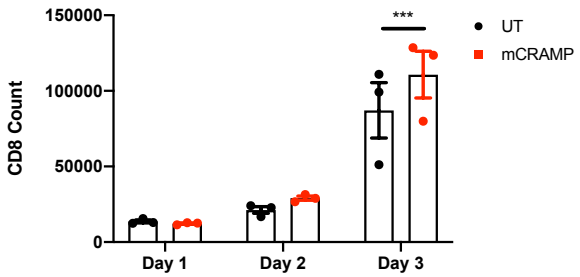


Figure 6.3: mCRAMP increases the total number of $CD8^+$ T cells cultured under non-lineage-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under non-lineage-driving conditions (αCD3 only, 5 μg/mL), with or without 2.5 μM synthetic mCRAMP, for up to 3 days. N = 3. Statistical significance (where *** represents < 0.001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

First, I sought to determine whether mCRAMP had any effect on $CD8^+$ T cell activation. Under non-lineage driving conditions, mCRAMP had no effect on the percentage of $CD8^+$ $CD62L^-$ $CD44^+$ T cells (**Figure 6.4 A**) but significantly increased the geometric mean of PD1 on days 1

and 3 (**Figure 6.4 B**). For example, this rose from 5033 (+/- 614) to 8586 (+/- 787) on day 1. There also appeared to be a trend indicating that PD1 expression was increased on day 2 (P = 0.08). These results are similar to those obtained for $CD4^+$ T helper cells exposed to mCRAMP, which also displayed an increase in PD1, even in the absence of polarizing cytokines.

Next, I sought to determine whether mCRAMP had any effect on cytokine production. The addition of mCRAMP to cells cultured in the absence of IL-17-inducing cytokines did not have any effect on IL-17A production by $CD8^+$ T lymphocytes (**Figure 6.4 C)**. However, cytotoxic $CD8^+$ T cells produce large quantities of IFNγ and a significant decrease in the percentage of $CD8^+$ $IFN\gamma^+$ T cells was observed on day 3 following exposure to mCRAMP: this dropped from 70.23% (+/- 3.89) to 57.20% (+/- 2.26) (**Figure 6.4 D**)[539].

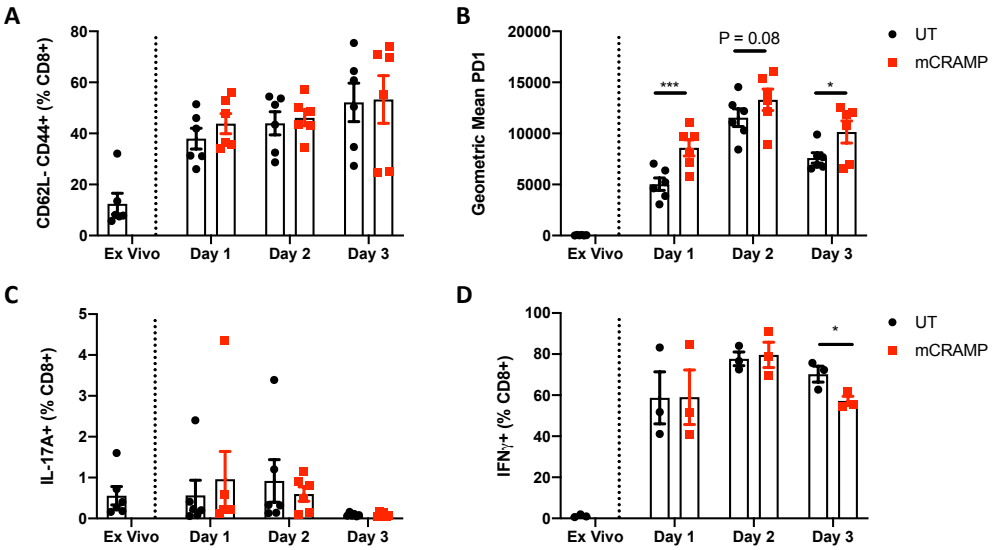


Figure 6.4: mCRAMP increases PD1 expression by $CD8^+$ T cells cultured under non-lineage driving conditions. Whole single cell splenic suspensions were cultured under non-lineage-driving conditions (αCD3 only, 5 μg/mL), with or without 2.5 μM synthetic mCRAMP, for up to 3 days **(A)** Percentages of $CD8^+$ $CD62L^-$ $CD44^+$ T cells, *ex vivo* and days 1-3 **(B)** Geometric mean of PD1 expression by $CD8^+$ T cells, *ex vivo* and days 1-3 **(C)** Percentages of $CD8^+$ $IL\text{-}17A^+$ T cells, *ex vivo* and days 1-3 **(D)** Percentages of $CD8^+$ $IFN\gamma^+$ T cells, ex vivo and days 1-3. Data shown is mean +/- standard error. N = 6. Statistical significance (where * represents < 0.05 and *** < 0.001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

6.3.4 mCRAMP increases the activation status of CD8⁺ T cells cultured under Tc17-driving conditions

The cytokine and molecular requirements for Tc17 differentiation are highly similar to those of CD4⁺ Th17 cells[531]. For instance, culturing CD8⁺ T lymphocytes in the presence of IL-6 and TGFβ induces the transcription of *Rorc* and the subsequent expression of IL-17[531].

mCRAMP increased PD1 expression by CD8⁺ T lymphocytes in the absence of polarizing cytokines but had no effect on IL-17 production, similarly to CD4⁺ T cells. However, mCRAMP increased the activation status and frequency of IL-17-producing CD4⁺ T cells cultured under Th17-driving conditions. I therefore sought to determine whether this host defence peptide had similar effects on CD8⁺ T lymphocytes in IL-17-inducing cultures *in vitro*. Whole single cell splenic suspensions were cultured under Tc17-polarizing conditions (IL-6, IL-23, TGFβ), as previously described, with or without 2 synthetic mCRAMP.

Figure 6.5 shows that mCRAMP had no significant effect on the total number of CD8⁺ T lymphocytes in Tc17 cultures.

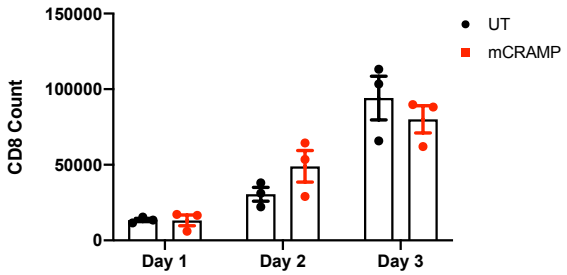


Figure 6.5: mCRAMP has no effect on the total number of CD8⁺ T cells cultured under Tc17-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under Tc17-driving conditions (20 ng/mL IL-6, 20 ng/mL IL-23 and 3 ng/mL TGFβ), with or without 2.5 μM synthetic mCRAMP, for up to 3 days. N = 3.

Almost 100% of CD8⁺ T cells were PD1⁺ by day 1 (data not shown). However, the geometric mean of PD1 expression was significantly increased following treatment with mCRAMP on days 1 to 3 (**Figure 6.6 C**). For example, on day 2, this rose by 72% from 7732 (+/- 713) to

13293 (+/- 614). Furthermore, the percentage of activated $CD8^+$ $CD62L^-$ $CD44^+$ T cells was also significantly increased following exposure to mCRAMP (**Figure 6.6 D**: day 2 UT: 44.37% +/- 8.39; day 2 mCRAMP: 55.12% +/- 8.43).

This indicates that mCRAMP increases the activation status of $CD8^+$ T cells cultured under Tc17-driving conditions, similarly to $CD4^+$ T lymphocytes.

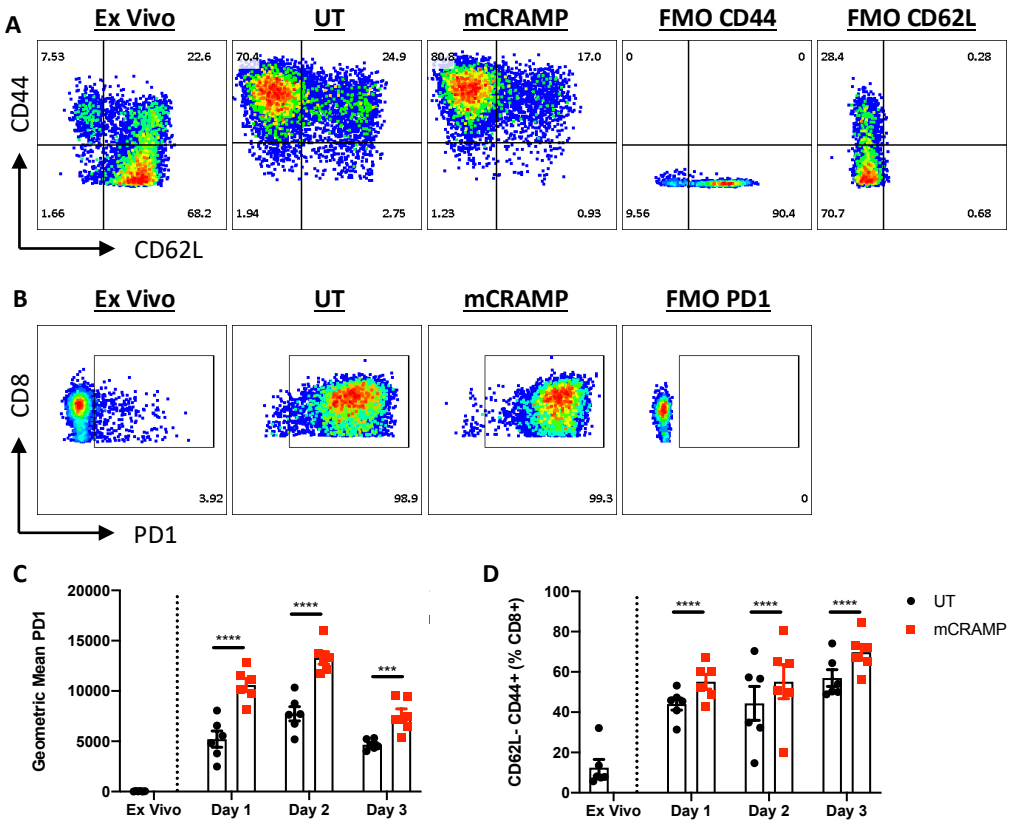


Figure 6.6: mCRAMP increases the activation status of $CD8^+$ T cells cultured under Tc17-driving conditions. Whole single cell splenic suspension from WT mice were cultured under Tc17-driving conditions, with or without 2.5 μM synthetic mCRAMP, for up to 3 days **(A)** Representative plots of CD44 and CD62L expression by $CD8^+$ T cells on day 2, assessed by flow cytometry **(B)** Representative plots of PD1 expression by $CD8^+$ T cells on day 2, assessed by flow cytometry **(C)** Geometric mean of PD1 expression by $CD8^+$ T cells, *ex vivo* and days 1-3 **(D)** Percentages of $CD62L^-$ $CD44^+$ T cells, *ex vivo* and days 1-3. Data shown is mean +/- standard error. N = 6. Statistical significance (where *** represents < 0.001 and **** < 0.0001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

6.3.5 mCRAMP enhances the development of IL-17-producing CD8⁺ T cells in Tc17 cultures

mCRAMP had no effect on the frequency of $CD8^+$ $IL\text{-}17A^+$ T lymphocytes when the cells were cultured under non-lineage-driving conditions. However, I have shown that mCRAMP increased RORγt and IL-17 expression by $CD4^+$ T lymphocytes when generated in the presence of IL-6, IL-23 and TGFβ[531]. To determine whether the peptide induced similar changes in $CD8^+$ T cells, I analysed the expression of RORγt and IL-17 by $CD8^+$ T cells by intracellular flow cytometry (**Figure 6.7**). Whole single cell splenic suspensions from WT mice were cultured under Tc17-driving conditions, as previously described, with or without synthetic mCRAMP.

Figure 6.7 demonstrates that mCRAMP promoted a significant increase, from 6.95% (+/- 0.74) to 21.42% (+/- 7.51), in the percentage of $CD8^+$ $ROR\gamma t^+$ T cells on day 1 (**Figure 6.7 C**). By day 2, this translated into a 1.56-fold increase, from 15.31% (+/- 1.34) to 23.94% (+/- 1.47), in the frequency of IL-17-producing $CD8^+$ T lymphocytes (**Figure 6.7 E**). Moreover, this increase in the proportion of $CD8^+$ $IL\text{-}17A^+$ T cells in response to mCRAMP was concentration-dependent, similarly to $CD4^+$ T lymphocytes (**Figure 6.7 G**). Interestingly, there was a significant decrease in the geometric mean of IL-17A of $CD8^+$ $IL\text{-}17A^+$ T cells, from 4115 (+/- 335) to 3857 (+/- 301) (**Figure 6.7 F**).

Taken together, these data indicate that mCRAMP increases the number of IL-17-producing $CD8^+$ T lymphocytes but decreases the amount of this cytokine produced by each cell.

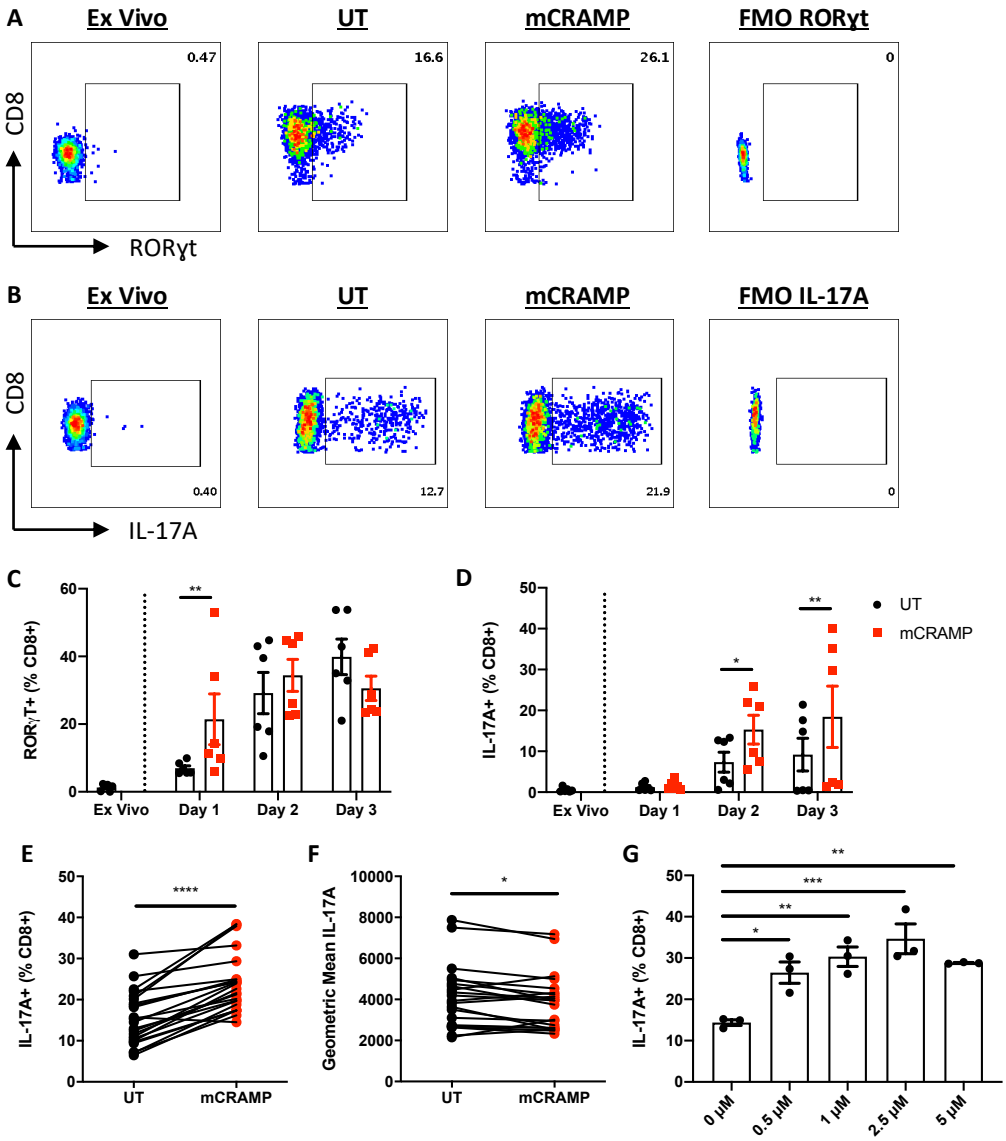


Figure 6.7: mCRAMP increases RORɣt expression and the frequency of IL-17A-producing CD8⁺ T cells cultured under Tc17-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under Tc17-driving conditions, with or without 2.5 μM synthetic mCRAMP, for up to 3 days **(A)** Representative plots RORɣt expression by CD8⁺ T cells on day 1, assessed by flow cytometry **(B)** Representative plots of IL-17A production by CD8⁺ T cells on day 2, assessed by flow cytometry **(C)** Percentages of CD8⁺ RORɣt⁺ T cells, *ex vivo* and days 1-3 (n = 6) **(D)** Percentages of CD8⁺ IL-17A⁺ T cells, *ex vivo* and days 1-3 (n = 6) **(E)** Percentages of CD8⁺ IL-17A⁺ T cells on day 2 (n = 23) **(F)** Geometric mean of IL-17A expression by CD8⁺ IL-17A⁺ T cells on day 2 (n = 23) **(G)** CD8⁺ IL-17A⁺ vs. mCRAMP dose response (n = 3). Data shown is mean +/- standard error. Statistical significance (where * represents < 0.05, ** < 0.01 and **** < 0.0001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test (C & D) or a paired t-test (E & F) or an ordinary one-way ANOVA with a Dunnett's multiple comparison post-test (G). UT: untreated.

In addition to IL-17A, several reports have shown that Tc17 cells also produce IL-17F and at percentages similar to $CD4^+$ T lymphocytes[126,540]. Furthermore, I previously demonstrated that mCRAMP preferentially enhanced the development of IL-17F-producing $CD4^+$ T cells in Th17 cultures. I therefore examined the effects of mCRAMP on IL-17F production by $CD8^+$ T lymphocytes cultured under Tc17-driving conditions, to determine whether the peptide exerted similar effects on this cell type (**Figure 6.8**).

Figure 6.8 shows that mCRAMP significantly increased the percentage of IL-17F single-positive $CD8^+$ T cells from 4.57% (+/- 0.98) to 7.92% (+/- 1.40) on day 2 (**Figure 6.8 C**). Moreover, mCRAMP significantly increased the percentage of IL-17A and IL-17F double-producing $CD8^+$ T lymphocytes from 5.83% (+/- 1.31) to 13.12% (+/- 2.08) (**Figure 6.8 D**). These observations were similar to those made for $CD4^+$ T helper cells (**Chapter 3: Figure 3.9**).

The percentage of IL-17A single-positive $CD8^+$ T lymphocytes was also significantly increased in mCRAMP-treated samples compared to untreated (**Figure 6.8 B**: 2.12% +/- 0.56 compared to 3.55% +/- 0.39). This was in contrast to $CD4^+$ T cells, whose IL-17A expression was not significantly affected by the peptide (**Chapter 3: Figure 3.9)**. These results therefore highlight a potential difference in the effects of mCRAMP on $CD8^+$ T lymphocytes compared to $CD4^+$ T helper cells.

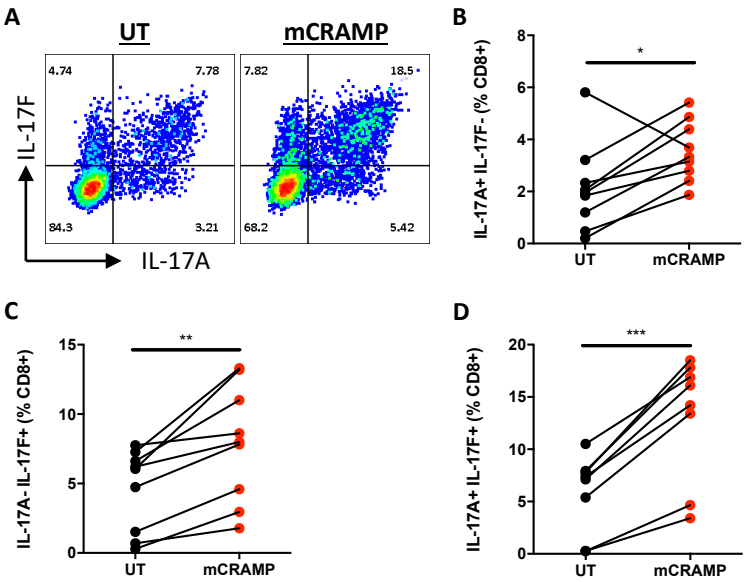


Figure 6.8: mCRAMP increases IL-17F production by CD8$^+$ T cells cultured under Tc17-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under Tc17-driving conditions, with or without 2.5 μM synthetic mCRAMP, for 2 days **(A)** Representative plots of IL-17A and IL-17F expression by CD8$^+$ T cells on day 2, assessed by flow cytometry **(B)** Percentages of CD8$^+$ IL-17A$^+$ IL-17F$^-$ T cells on day 2 **(C)** Percentages of CD8$^+$ IL-17A$^-$ IL-17F$^+$ T cells on day 2 **(D)** Percentages of CD8$^+$ IL-17A$^+$ IL-17F$^+$ T cells on day 2 . Data shown is mean +/- standard error. N = 6. Statistical significance (where * represents < 0.05. ** < 0.01 and *** < 0.001) was determined using a paired t-test. UT: untreated.

6.3.6 mCRAMP acts via the aryl hydrocarbon receptor to enhance Tc17 differentiation

I have previously shown that mCRAMP upregulated the expression of AHR in $CD4^+$ T lymphocytes and that this specifically promoted the development of $CD4^+$ $IL\text{-}17A^+$ $IL\text{-}17F^+$ T cells (**Chapter 4: Figures 4.6 & 4.7**).

To determine whether a similar AHR-dependent mechanism could be seen in $CD8^+$ T cells, I cultured whole single cell splenic suspensions from WT mice under Tc17-driving conditions, as previously described, with or without synthetic mCRAMP, and examined the expression of AHR by intracellular flow cytometry (**Figure 6.9**).

Figure 6.9 B shows that mCRAMP significantly increased the percentage of $CD8^+$ AHR^+ T cells on day 1, from 12.57% (+/- 0.73) to 18.73% (+/- 0.30), although no differences were observed on day 2. This is in contrast to $CD4^+$ T cells, who displayed a significant and cumulative increase over time (**Chapter 4: Figure 4.6**).

Hayes and colleagues suggested that the AHR pathway plays a greater role in Th17 development compared to Tc17[541]. Nonetheless, to determine whether the effects of mCRAMP on the differentiation of Tc17 cells were AHR-dependent, I cultured whole single cell splenic suspensions under Tc17-driving conditions for 2 days, as previously described, with or without synthetic mCRAMP and/or an AHR antagonist (CH223191)[447].

mCRAMP significantly increased the proportion of IL-17A single-positive cells from 2.05% (+/- 0.11) to 3.78% (+/- 0.38). However, this was abolished (2.27% +/- 0.29) following addition of the AHR antagonist (**Figure 6.9 C**). CH223191 also prevented the increase in the percentage IL-17A and IL-17F double-producing $CD8^+$ T cells normally induced by mCRAMP (**Figure 6.9 D**). Conversely, the AHR antagonist did not appear to have a significant effect on the frequency of $CD8^+$ $IL\text{-}17A^-$ $IL\text{-}17F^+$ T cells (**Figure 6.9 E**).

These results indicate that mCRAMP acts via AHR to promote the development of $CD8^+$ $IL\text{-}17A^+$ $IL\text{-}17F^+$ T lymphocytes. However, unlike in $CD4^+$ T helper cells, mCRAMP also acts via this transcription factor to boost the differentiation of IL-17A single-positive Tc17s.

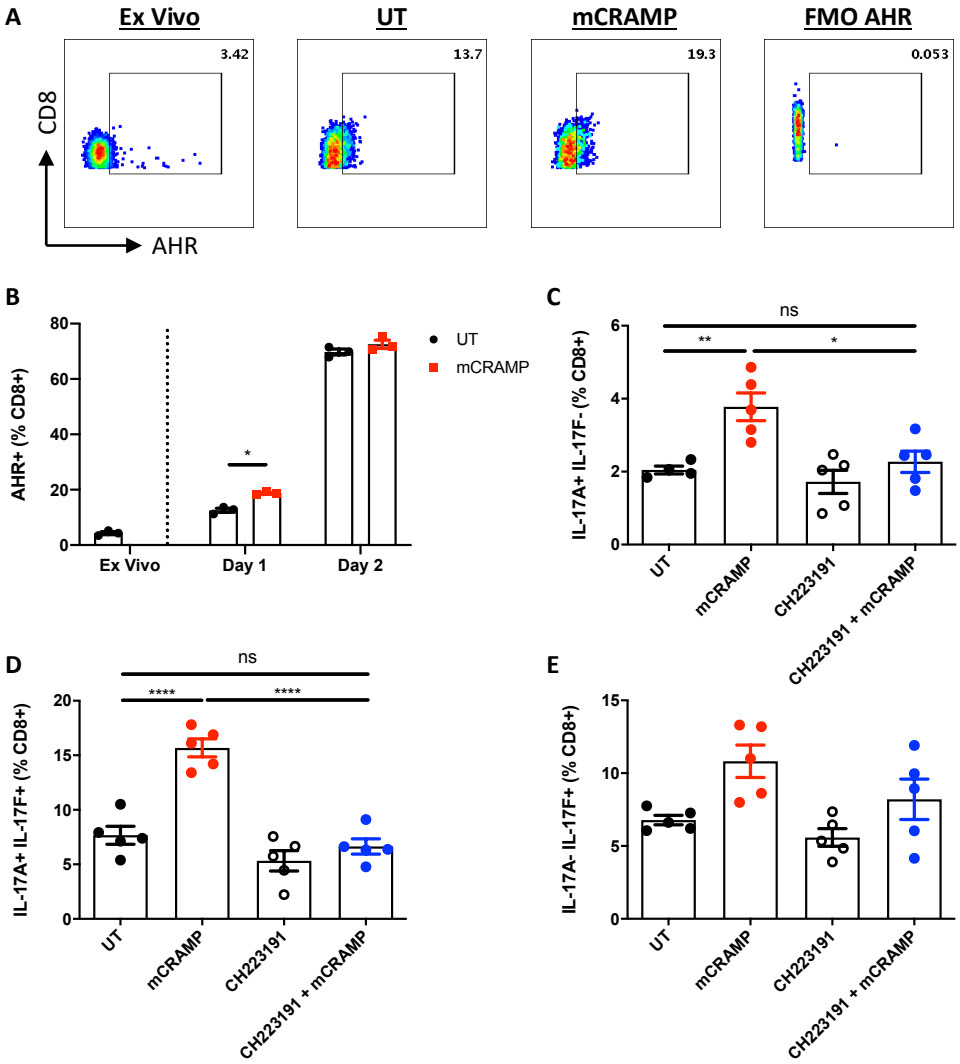


Figure 6.9: CH223191 prevents the increase in the percentage of CD8$^+$ IL-17A$^+$ IL-17F$^-$ and IL-17A$^+$ IL-17F$^+$ T cells induced by mCRAMP. Whole single cell splenic suspensions were cultured under Tc17-driving conditions for 2 days, with or without 2.5 μM synthetic mCRAMP and/or an AHR antagonist (CH223191, 10 μM) **(A)** Representative plots of AHR expression production by CD8$^+$ T cells on day 1, assessed by flow cytometry **(B)** Percentages of CD8$^+$ AhR$^+$ T cells, *ex vivo* and days 1-2 (n = 3) **(C)** Percentages of CD8$^+$ IL-17A$^+$ IL-17F$^-$ T cells on day 2 (n = 5) **(D)** Percentages of CD8$^+$ IL-17A$^+$ IL-17F$^+$ T cells on day 2 (n = 5) **(E)** Percentages of CD8$^+$ IL-17A$^-$ IL-17F$^+$ T cells on day 2 (n = 5). Data shown is mean +/- standard error. Statistical significance (where * represents < 0.05, ** < 0.01 and **** < 0.0001) was determined using an ordinary one-way ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

6.3.7 mCRAMP acts directly on CD8⁺ T cells to enhance Tc17 differentiation in vitro

I have shown that mCRAMP had no significant effect on the phenotype of differentiated DCs and that the peptide could act directly on CD4⁺ T cells to increase the frequency of CD4⁺ IL-17A⁺ T lymphocytes in IL-17-inducing cultures (**Chapter 3: section 3.3.8**). To determine whether mCRAMP was capable of acting directly on CD8⁺ T lymphocytes, I cultured sorted splenic CD8⁺ T cells (DAPI⁻ CD4⁻ CD8⁺; purity > 97%) under Tc17-driving conditions, as previously described, with or without synthetic peptide (**Figure 6.10**).

Figure 6.10 shows that mCRAMP significantly increased the proportion of CD8⁺ IL-17A⁺ T lymphocytes from 0.25% (+/- 0.09) to 1.46% (+/- 0.13) (**Figure 6.10 B**). The frequencies of IL-17-producing CD8⁺ T lymphocytes generated were substantially lower than those obtained from culturing whole single cell splenic suspensions. This could indicate that CD8⁺ T cells require additional signals or growth factors that are provided by other cell types present within the spleen. Nonetheless, the concentration of IL-17A in cell culture supernatants measured by ELISA, rose 10-fold from 20 to 269 pg/mL, following exposure to mCRAMP (**Figure 6.10 C**).

Taken together, these results suggest that mCRAMP can act directly on CD8⁺ T cells to enhance Tc17 differentiation.

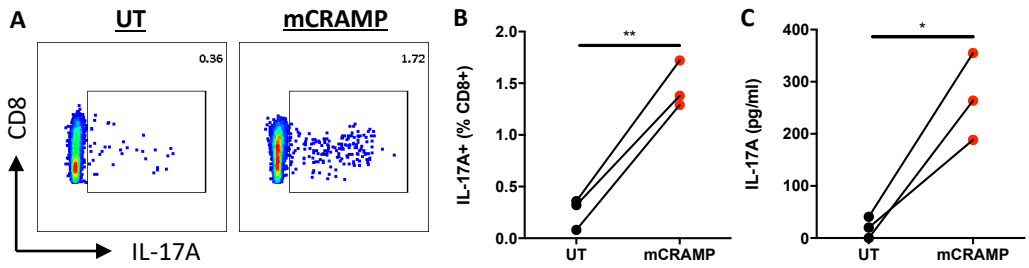


Figure 6.10: mCRAMP acts directly on CD8⁺ T cells. Sorted CD8⁺ T cells were cultured under Tc17-driving conditions for 2 days, with or without 2.5 μM synthetic mCRAMP **(A)** Representative plots of IL-17A production by CD8⁺ T cells on day 2, assessed by flow cytometry **(B)** Percentages of CD8⁺ IL-17A⁺ T cells on day 2 **(C)** Concentration of IL-17A in cell culture supernatants on day 2, determined by ELISA. Data shown is mean +/- standard error. N = 3. Statistical significance (where * represents < 0.05 and ** < 0.01) was determined using a paired t-test. UT: untreated.

6.3.8 mCRAMP increases the frequency of CD8$^+$ IL-23R$^+$ and IL-6R$^+$ T cells cultured under Tc17-driving conditions

mCRAMP could act directly on CD8$^+$ T cells to increase the development of IL-17-producing CD8$^+$ T lymphocytes. One possible mechanism through which the peptide could achieve this is by upregulating the expression of receptors required for Tc17 differentiation, thereby increasing the sensitivity of CD8$^+$ T cells to IL-17-inducing cytokines. I therefore analysed the expression of IL-23R and IL-6R by CD8$^+$ T lymphocytes following culture under Tc17-driving conditions, with or without synthetic mCRAMP (**Figure 6.11**). The lack of a reliable antibody that can be used to detect TGFβR by flow cytometry prevented the analysis of CD8$^+$ TGFβR$^+$ T cells.

Similarly to those obtained from CD4$^+$ T helper cells, the results were highly variable. However, a significant increase in the percentage of CD8$^+$ IL-23R$^+$ (**Figure 6.11 C**: 19.39% +/- 8.34 compared to 11.72% +/- 4.94) and CD8$^+$ IL-6R$^+$ T cells (**Figure 6.11 D**: 6.98% +/- 1.05 compared to 3.46% +/- 0.39) was observed in response to mCRAMP on days 2 and 1, respectively.

These results need to be confirmed by PCR (and should also include TGFβR) but they suggest that mCRAMP increases the expression of IL-6R and IL-23R by CD8$^+$ T lymphocytes in Tc17 cultures. This data is in contrast to CD4$^+$ T lymphocytes, which did not display any significant differences in IL-6R or IL-23R, and therefore adds to the differential roles previously noted for the peptide on CD4$^+$ and CD8$^+$ T cells.

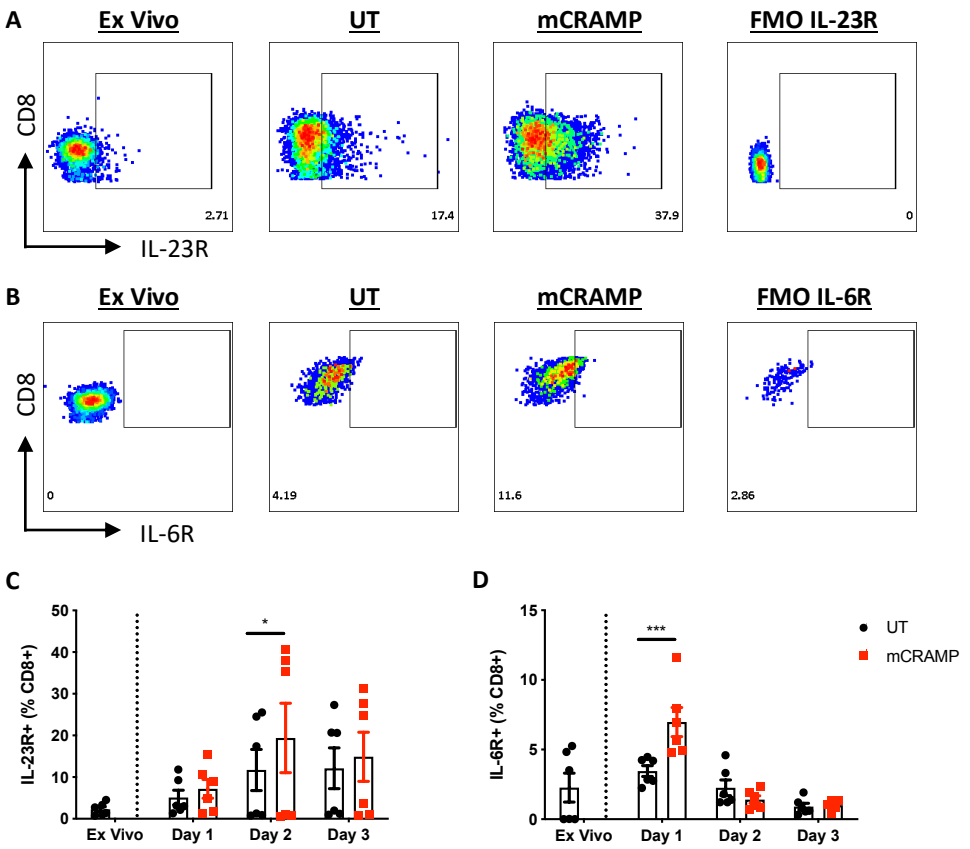


Figure 6.11: mCRAMP increases the expression of IL-6R and IL-23R by CD8⁺ T cells cultured under Tc17-driving conditions. Whole single cell splenic suspensions from WT mice were cultured under Tc17-driving conditions, with or without 2.5 µM synthetic mCRAMP **(A)** Representative plots of IL-23R expression by CD8⁺ T cells on day 2, assessed by flow cytometry **(B)** Representative plots of IL-6R⁺ expression by CD8⁺ T cells on day 1, assessed by flow cytometry **(C)** Percentages of CD8⁺ IL-23R⁺ T cell, *ex vivo* and days 1-3 **(D)** Percentages of CD8⁺ IL-6R⁺ T cell, *ex vivo* and days 1-3. Data shown is mean +/- standard error. N = 6. Statistical significance (where * represents < 0.05 and *** < 0.001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

6.3.9 mCRAMP decreases T-bet expression and IFNγ production by CD8⁺ T lymphocytes cultured under Tc17-driving conditions

Gene expression analysis of $CD4^+$ T cells cultured under Th17-driving conditions revealed that several Th1-related genes were significantly downregulated following exposure to mCRAMP (**Chapter 4: Figure 4.1**). I subsequently found that mCRAMP significantly decreased the frequency of $CD4^+$ T-bet^+ and $CD4^+$ $IFN\gamma^+$ T cells, suggesting that the peptide skews T helper cell differentiation away from the Th1 lineage (**Chapter 4: Figure 4.5**).

Like Th1 lymphocytes, Tc1 cells are significant producers of IFNγ and their differentiation is driven, in part, by the master transcriptional regulator T-bet[529]. I therefore investigated the expression T-bet and IFNγ by $CD8^+$ T cells cultured under Tc17-driving conditions, with or without synthetic mCRAMP, to determine whether the peptide also skews cytotoxic $CD8^+$ T cell differentiation (**Figure 6.12**).

Figure 6.12 shows that mCRAMP significantly decreased T-bet expression on days 2 and 3 (**Figure 6.12 C**). For example, the percentage of $CD8^+$ $ROR\gamma t^-$ T-bet^+ T cells dropped from 48.97% (+/- 9.99) to 39.88% (+/- 9.12) on day 2.

A subset of Tc17 cells has been shown to co-express T-bet and RORγt under certain conditions[542–544]. Similarly to $CD4^+$ T lymphocytes, I observed a significant decrease in the frequency of $CD8^+$ $ROR\gamma t^+$ T-bet^+ T cells on day 3 following exposure to mCRAMP, from 29.92% (+/- 2.70) to 20.65% (+/- 3.79) (**Figure 6.12 D**).

The reduction in T-bet expression by $CD8^+$ T cells cultured in the presence of mCRAMP was associated with a significant decrease in IFNγ production: the percentage of $CD8^+$ $IFN\gamma^+$ T cells fell from 22.84% (+/- 1.90) to 16.58% (+/- 1.48) on day 2 (**Figure 6.12 E**). No differences in the geometric mean of IFNγ of $CD8^+$ $IFN\gamma^+$ T cells were observed (**Figure 6.12 F**) and no IL-17/IFNγ double-producing lymphocytes were identified (data not shown).

Taken together, these results support the hypothesis that like $CD4^+$ T helper cells, mCRAMP skews cytotoxic T cell polarisation away from a Tc1 phenotype and towards the Tc17 lineage.

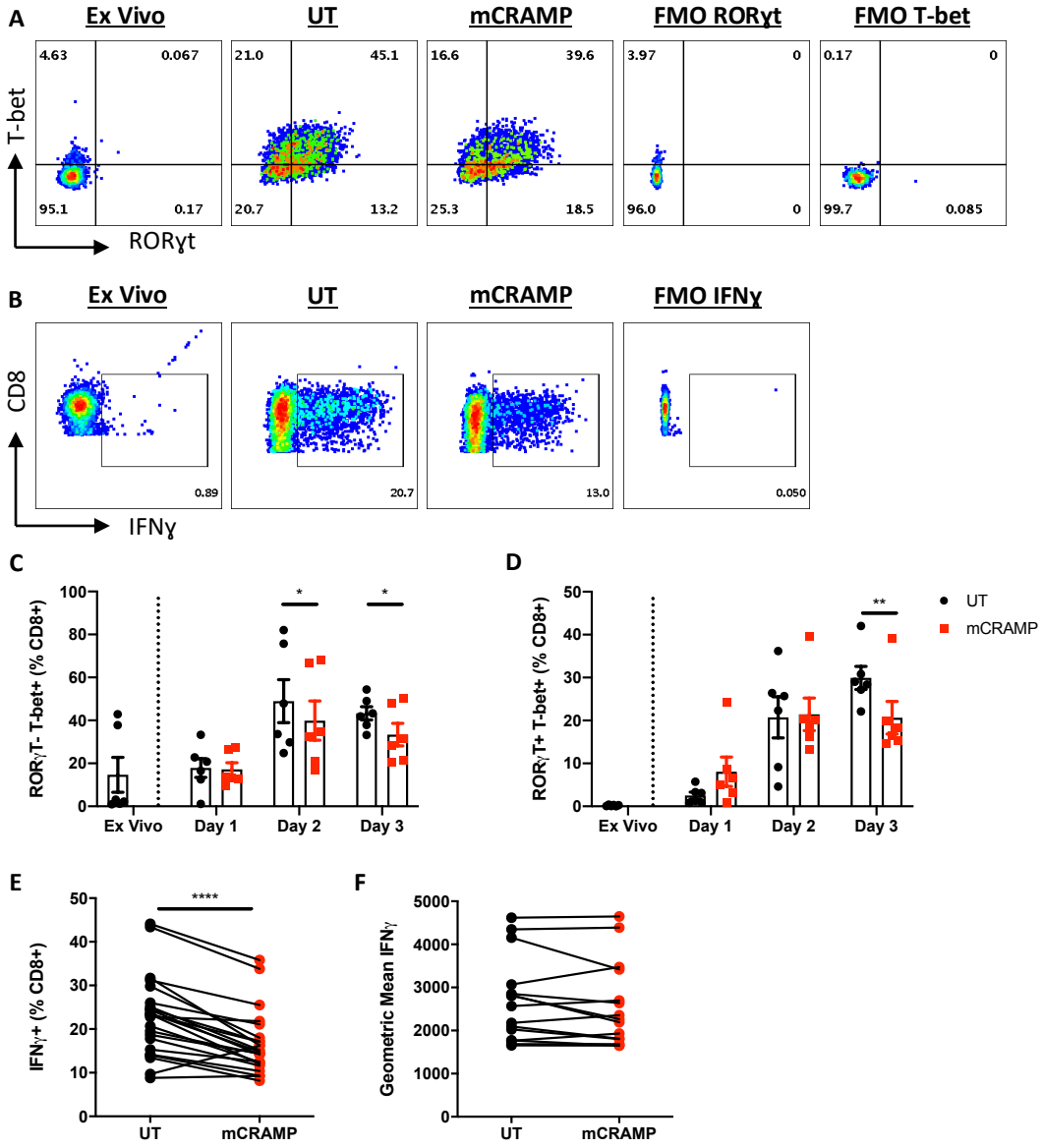


Figure 6.12: mCRAMP decreases T-bet expression and IFNɣ production in CD8⁺ T cells cultured under Tc17-driving conditions. Whole single cell splenic suspensions were cultured under Tc17-driving conditions, with or without 2.5 µM synthetic mCRAMP **(A)** Representative plots of RORγt and T-bet expression by CD8⁺ T cells on day 2, assessed by flow cytometry **(B)** Representative plots of IFNɣ production by CD8⁺ T cells, assessed by flow cytometry **(C)** Percentages of CD8⁺ RORγt⁻ T-bet⁺ T cells, *ex vivo* and days 1-3 (n = 6) **(D)** Percentages of CD8⁺ RORγt⁺ T-bet⁺ T cells, *ex vivo* and days 1-3 (n = 6) **(E)** Percentages of CD8⁺ IFNɣ⁺ T cells on day 2 (n = 21) **(F)** Geometric mean of IFNγ expression by CD8⁺ IFNγ⁺ T cells. Data shown is mean +/- standard error. Statistical significance (where * represents < 0.05, ** < 0.01 and **** < 0.0001) was determined using two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test (C & D) or a paired t-test (E). UT: untreated.

6.3.10 mCRAMP increases CD8$^+$ T cell viability cultured under Tc17-driving conditions

One of the potential mechanisms through which mCRAMP could increase Tc17 differentiation is by promoting the survival of CD8$^+$ IL-17$^+$ T cells and/or the death of non-Tc17 subsets. I therefore performed an annexin/PI apoptosis assay to examine cell death in non-lineage-driving and Tc17 cultures treated with or without synthetic mCRAMP (**Figure 6.13**). As discussed previously, it was only possible to analyse the CD8$^+$ T cell population as a whole using this method.

Figure 6.13 D shows there was a trend suggesting that mCRAMP increased the percentage of CD8$^+$ annexin$^-$ PI$^-$ ("alive") T cells when they were cultured under non-lineage-driving conditions in the absence of polarising cytokines, similarly to CD4$^+$ T lymphocytes. However, this was not statistically significant. On the other hand, mCRAMP significantly decreased the percentage of CD8$^+$ annexin$^+$ PI$^+$ ("necrotic") T cells on days 1, 2 and 3 (**Figure 6.13 H**). For instance, the frequency of CD8$^+$ annexin$^+$ PI$^+$ T cells dropped from 19.73% (+/- 1.79) to 14.40% (+/- 0.62) on day 2. No differences in the percentage of apoptotic (annexin$^+$ PI$^-$) T cells were observed between untreated and mCRAMP-treated samples (**Figure 6.13 F**). It is important to note to that an increase in the total number of CD8$^+$ T lymphocytes in non-polarising cultures exposed to mCRAMP was observed on day 3 (**Figure 6.13 B**). As a result, it is difficult to conclude whether or not these differences were responsible for the increase in the total number of CD8$^+$ T cells, or simply a result of increased proliferation.

mCRAMP had no effect on the total number of CD8$^+$ T cells following culture under Tc17-driving conditions (**Figure 6.13 C**). However, mCRAMP significantly increased the percentage of CD8$^+$ annexin$^-$ PI$^-$ (**Figure 6.13 E**) and decreased the percentage of CD8$^+$ annexin$^+$ PI$^+$ T cells (**Figure 6.13 I**), but only on day 2. More specifically, mCRAMP increased the proportion of CD8$^+$ annexin$^-$ PI$^-$ T cells from 69.13% (+/- 4.51) to 74.32% (+/- 4.14), and decreased CD8$^+$ annexin$^+$ PI$^+$ from 14.78% (+/- 2.74) to 9.79% (+/- 2.30). No differences in the percentage of CD8$^+$ annexin$^+$ PI$^-$ T cells were observed following exposure to mCRAMP (**Figure 6.13 G**).

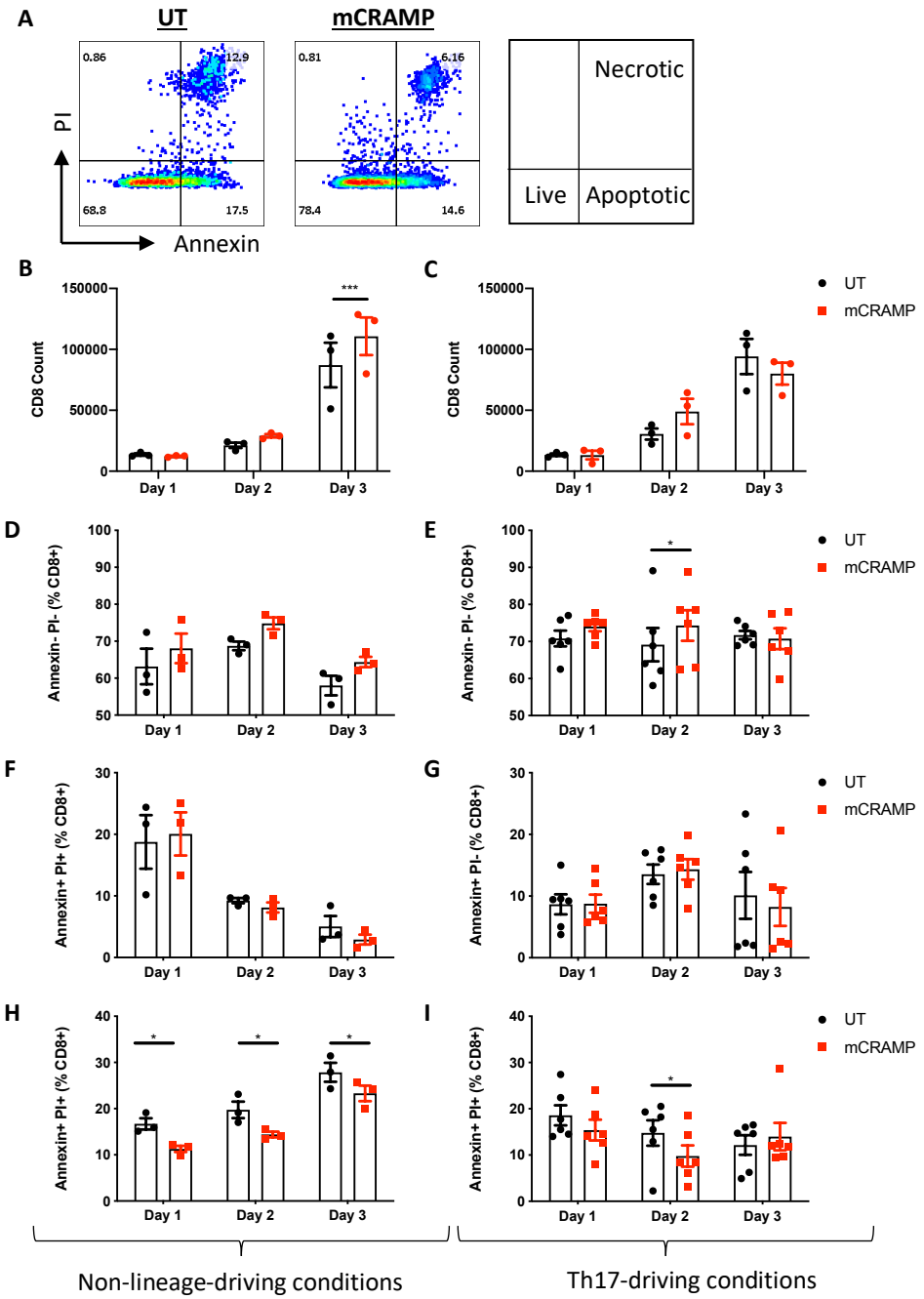


Figure 6.13: mCRAMP increases the viability of CD8⁺ T cells. Whole single cell splenic suspensions were cultured under non-lineage-driving or Tc17-polarizing conditions, with or without 2.5 µM synthetic mCRAMP **(A)** Representative plots of annexin/PI staining of CD8⁺ T cells on day 2, assessed by flow cytometry **(B)** Total numbers of CD8⁺ T cells in non-lineage driving cultures **(C)** Total numbers of CD8⁺ T cells in Th17 cultures **(D)** Percentages of CD8⁺ annexin⁻ PI⁻ T cells on days 1-3 following culture under non-lineage-driving conditions (n = 3) **(E)** Percentages of CD8⁺ annexin⁻ PI⁻ T cells on days 1-3 following culture under Th17-driving conditions (n = 6) **(F)** Percentages of CD8⁺ annexin⁺ PI⁻ T cells on days 1-3 following culture under non-lineage-driving conditions (n = 3) **(G)** Percentages of CD8⁺ annexin⁺ PI⁻ T cells on days 1-3 following culture under Th17-driving conditions (n = 6) **(H)** Percentages of CD8⁺ annexin⁺ PI⁺ T cells on days 1-3 following culture under non-lineage-driving conditions (n = 3) **(I)** Percentages of CD8⁺ annexin⁺ PI⁺ T cells on days 1-3 following culture under Th17-driving conditions (n = 6). Data shown is mean +/- standard error. Statistical significance (where * represents < 0.05 and *** < 0.001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

Next, I sought to examine the effects of mCRAMP on the viability of $CD8^+$ IL-$17A^+$ and $CD8^+$ $IFN\gamma^+$ T lymphocytes by analysing the uptake of a fixable live/dead stain (**Figure 6.14)**. Using this method, I observed a significant decrease, from 14.35% (+/- 1.14) to 8.30% (+/- 0.79), in the percentage of dead $CD8^+$ T cells on day 2 (**Figure 6.14 B**). This supports the results obtained from the annexin/PI apoptosis assay (on day 2), as well as the hypothesis that mCRAMP protects the $CD8^+$ T cell population as a whole from death.

Furthermore, mCRAMP significantly decreased the percentage of dead $CD8^+$ IL-$17A^+$ T lymphocytes from 9.36% (+/- 1.15) to 4.75% (+/- 0.72) (**Figure 6.14 D**). Interestingly, the percentage of dead $CD8^+$ $IFN\gamma^+$ T cells in Tc17 cultures was also significantly reduced following exposure to mCRAMP (**Figure 6.14 F:** 3.92% +/- 0.58 compared to 5.63% +/- 0.71). This was in contrast to $CD4^+$ $IFN\gamma^+$ T helper cells who were not protected from death.

The results obtained from the annexin/PI apoptosis assay do not provide a clear answer as to whether or not mCRAMP enhances the survival of $CD8^+$ T lymphocytes similarly to $CD4^+$ T helper cells. The fact that significant differences were only observed on day 2 could indicate that these effects are not biologically relevant. However, the data generated by looking at the uptake of a live/dead stain imply that mCRAMP acts as a survival factor to increase the viability of all $CD8^+$ T cells, whether they be IL-17^+ or not. Further investigation (such as using cell counts instead of percentages) will be required to help clarify these results.

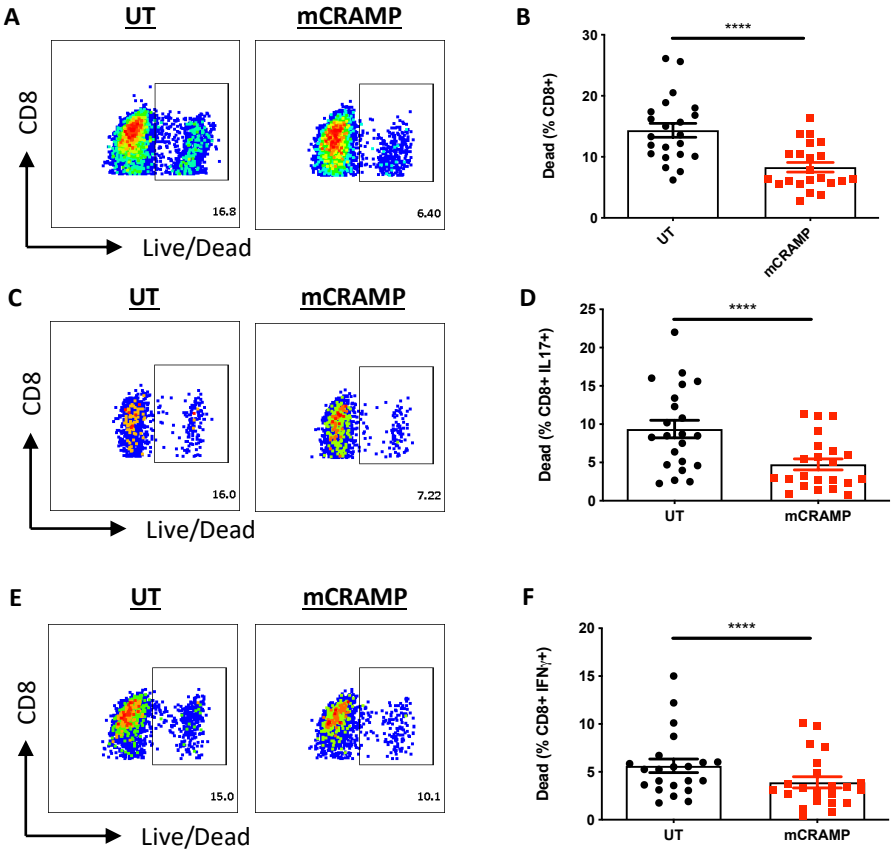


Figure 6.14: mCRAMP promotes the survival of CD8⁺ IL-17⁺ and IFNɣ⁺ T cells. Whole single cell splenic suspensions were cultured under Tc17-driving conditions, with or without 2.5 µM synthetic mCRAMP **(A)** Representative plots of dead CD8⁺ T cells on day 2 (gated on single, CD8⁺ lymphocytes), assessed by flow cytometry **(B)** Percentages of dead CD8⁺ T cells on day 2 **(C)** Representative plots of dead CD8⁺ IL-17A⁺ T cells on day 2 (gated on single lymphocytes that are CD8⁺ IL-17A⁺), assessed by flow cytometry **(D)** Percentages of dead CD8⁺ IL-17A⁺ T cells on day 2 **(E)** Representative plots of dead CD8⁺ IFNɣ⁺ T cells on day 2 (gated on single lymphocytes that are CD8⁺ IFNɣ⁺), assessed by flow cytometry **(F)** Percentages of dead CD8⁺ IFNɣ⁺ T cells on day 2. Data shown is mean +/- standard error. N = 22. Statistical significance (where **** represents < 0.0001) was determined using a paired t-test. UT: untreated.

6.3.11 mCRAMP increases the proliferation of CD8$^+$ T cells cultured under Tc17-driving conditions

An apparent increase in Tc17 differentiation could be explained, in part, by an increase in the proliferation of CD8$^+$ IL-17$^+$ T lymphocytes. To address this, I monitored the proliferation of these cells in IL-17-inducing cultures by CFSE dye dilution (**Figure 6.15**).

Figure 6.15 shows that there was a significant decrease in the geometric mean of CFSE of total CD8$^+$ T lymphocytes, suggesting that mCRAMP increases the proliferation of the CD8$^+$ T cell population as a whole (**Figure 6.15 B**). This was in contrast to CD4$^+$ T helper lymphocytes, which did not display any differences in their proliferative capacity.

Due to high levels of cell death in stimulated, intracellular cytokine- and CFSE-stained cultures, I analysed the division of CD8$^+$ RORγt$^+$ and T-bet$^+$ T lymphocytes to monitor the proliferation of Tc1 and Tc17 cells. The proliferation of CD8$^+$ RORγt$^+$ T cells was not significantly different between untreated and mCRAMP-treated samples (**Figure 6.15 D**). However, there was a trend suggesting that the geometric mean of CFSE of CD8$^+$ RORγt$^+$ T cells was decreased following exposure to mCRAMP, similarly to the whole CD8$^+$ population. Nonetheless, these results imply that mCRAMP does not increase the proliferation of CD8$^+$ RORγt$^+$ T cells to boost Tc17 differentiation.

On the other hand, the geometric mean of CFSE of CD8$^+$ T-bet$^+$ T cells was significantly increased following exposure to mCRAMP (**Figure 6.15 F**). This was also in contrast to CD4$^+$ T lymphocytes and suggests that mCRAMP inhibits the proliferation of CD8$^+$ T-bet$^+$ T cells, which could account, in part, for the decrease in their frequency.

These results indicate that mCRAMP increases the proliferation of the CD8$^+$ T cell population as a whole but that this is not a result of enhanced proliferation of CD8$^+$ RORγt$^+$ T lymphocytes. This insinuates that there must be another subset of CD8$^+$ T cells (not Tc1/Tc17) that are proliferating more than normal in response to mCRAMP.

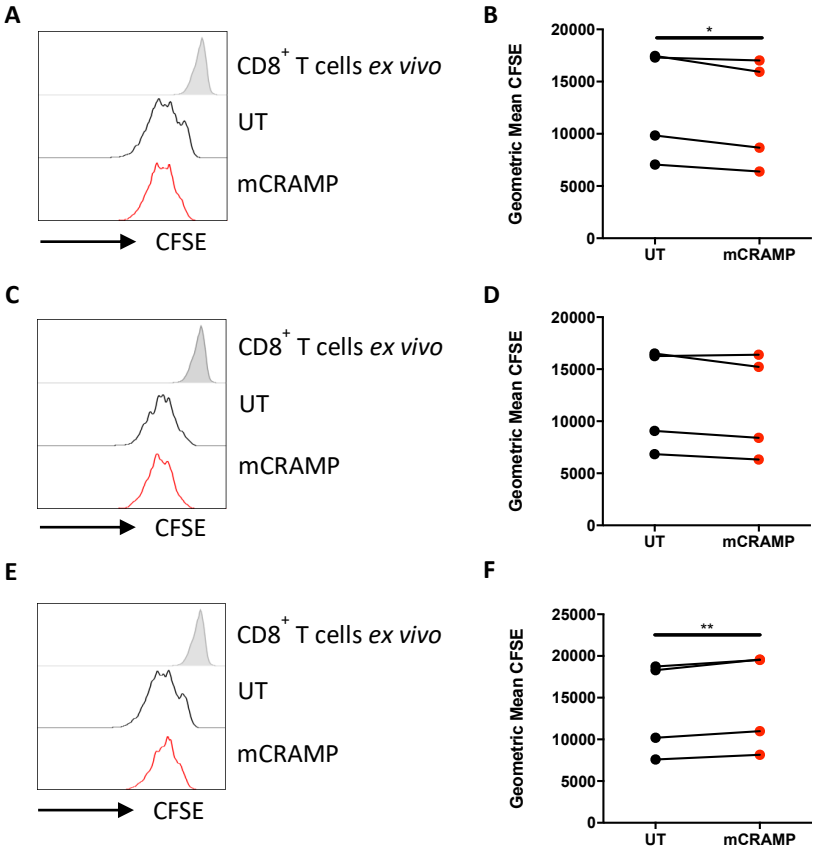


Figure 6.15: mCRAMP increases CD8⁺ T cell proliferation. Whole single cell splenic suspensions were cultured under Tc17-driving conditions for 2 days, with or without 2.5 μM synthetic mCRAMP **(A)** Representative CFSE plot of CD8⁺ T cells, assessed by flow cytometry **(B)** Geometric mean of CD8⁺ CFSE⁺ T cells on day 2 **(C)** Representative CFSE plot of CD8⁺ RORɣt⁺ T cells, assessed by flow cytometry **(D)** Geometric mean of CD8⁺ RORɣt⁺ CFSE T cells on day 2 **(E)** Representative CFSE plot of CD8⁺ T-bet⁺ T cells, assessed by flow cytometry **(F)** Geometric mean of CD8 T-bet⁺ CFSE T cells on day 2. Data shown is mean +/- standard error. N = 4. Statistical significance (where * represents < 0.05 and ** < 0.01) was determined using a paired t-test. UT: untreated.

6.3.12 mCRAMP requires TGFβ to enhance Tc17 differentiation in vitro

mCRAMP increased PD1 expression by $CD8^+$ T lymphocytes when cultured under non-lineage-driving and Tc17-polarising conditions. However, the peptide only increased the frequency of $CD8^+$ $IL\text{-}17A^+$ T cells when they were generated in the presence of Tc17-polarizing cytokines. This suggests that, like $CD4^+$ T cells, there are at least two, separate T cell signalling pathways induced by mCRAMP, one of which requires IL-6, IL-23 and/or TGFβ.

mCRAMP required TGFβ to boost the differentiation of $CD4^+$ Th17 cells (**Chapter 3: Figure 3.19**). To examine the requirement for TGFβ in $CD8^+$ Tc17 responses, I cultured whole single cell splenic suspensions with each cytokine alone, or by systematically omitting each one at a time, with or without synthetic mCRAMP (**Figure 6.16**).

Figure 6.16 shows that the geometric mean of RORγt expression was not significantly different between UT and mCRAMP-treated samples when the cells were cultured in the absence of TGFβ (**Figure 6.16 A**). This suggests that mCRAMP requires TGFβ to promote Tc17 differentiation, similarly to $CD4^+$ Th17 lymphocytes.

Accordingly, mCRAMP failed to significantly increase the percentage of $CD8^+$ $ROR\gamma t^+$ T cells when the cells were cultured with IL-6 and IL-23 (in the absence of TGFβ) or with IL-23 alone (**Figure 6.16 B**). Furthermore, mCRAMP increased the percentage of $CD8^+$ $ROR\gamma t^+$ T cells, from 14.56% (+/- 1.80) to 28.19% (+/- 1.01), when generated in the presence of just TGFβ. However, a significant increase in the percentage of $CD8^+$ $ROR\gamma t^+$ T cells was observed following exposure to mCRAMP when the cells were cultured with IL-6 alone. There was also a trend suggesting a similar increase in IL-23-stimulated cultures ($p = 0.16$) This could be explained by an increasing number of $CD8^+$ T cells transiently expressing very low levels of RORγt, which is likely insufficient to have any downstream effects on IL-17 production.

An increase in RORγt expression did not necessarily translate into IL-17 production (**Figure 6.16 C**). Overexpression of RORγt in $CD8^+$ T lymphocytes under non-polarizing conditions induces only low frequencies of IL-17-producing cells, indicating that RORγt is necessary but not sufficient for IL-17 expression[531]. Furthermore, similarly to in $CD4^+$ T helper cells, several

other transcription factors act cooperatively to promote Tc17 differentiation and IL-17 production[528]. For example, IRF4 controls Tc17 development by balancing the levels of RORγt, Eomes and FOXP3[532]. It is therefore likely that all three Tc17-polarizing cytokines are required to provide an optimal environment in which mCRAMP can increase the expression of this cytokine.

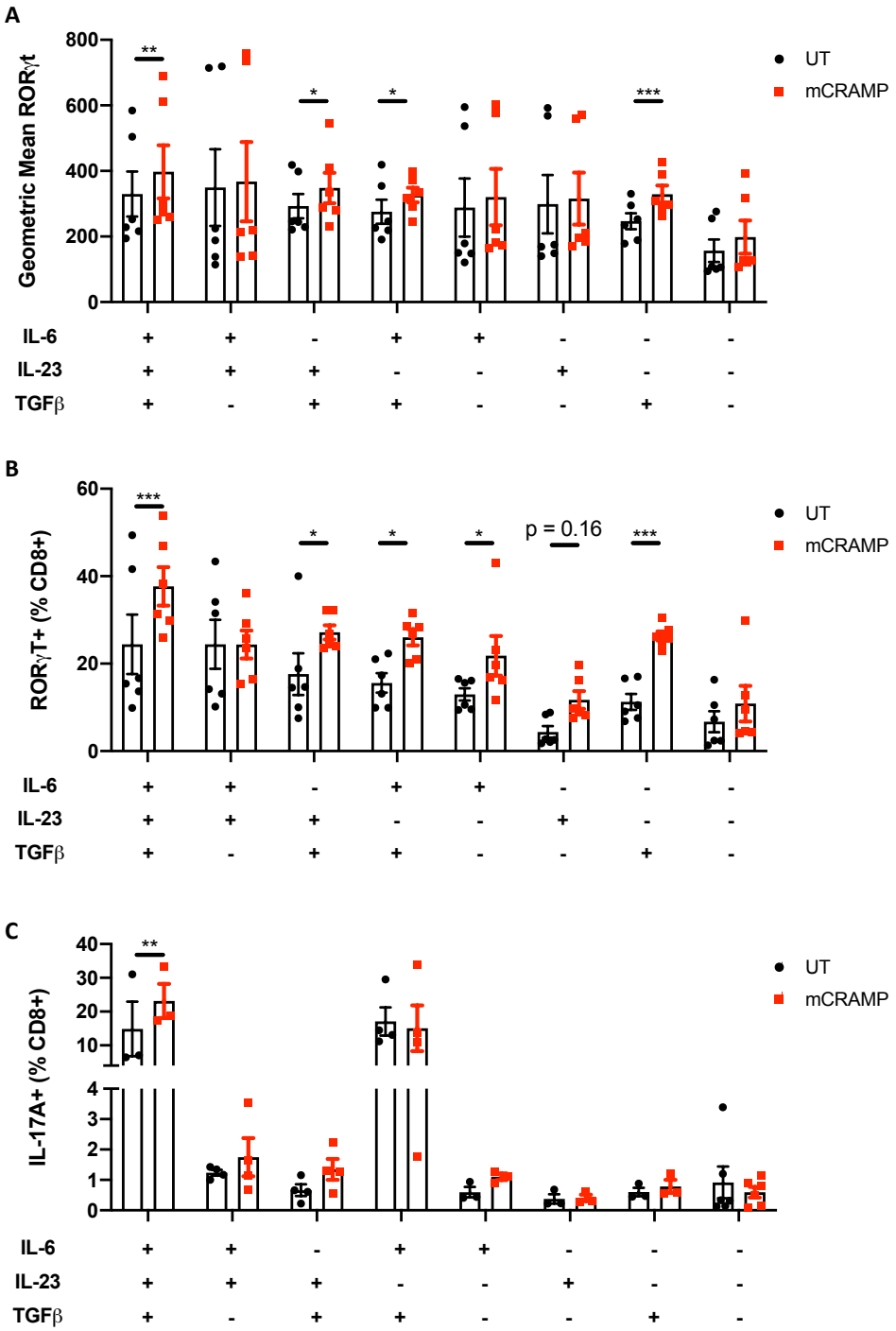


Figure 6.16: mCRAMP fails to increases RORɣt expression in CD8⁺ T cells in the absence of TGFβ. Whole single cell splenic suspensions were cultured with different combinations of IL-6 (20 ng/mL), IL-23 (20 ng/mL) and TGFβ (3 ng/mL), with or without 2.5 µM synthetic mCRAMP **(A)** Geometric mean of RORɣt expression by CD8⁺ T cells on day 1 **(B)** Percentages of CD8⁺ RORɣt⁺ T cells on day 1 **(C)** Percentages of CD8⁺ IL-17A⁺ T cells on day 2. Data shown is mean +/- standard error. N = 6. Statistical significance (where * represents < 0.05, ** < 0.01 and *** < 0.001) was determined using a two-way repeated measures ANOVA with a Bonferroni multiple comparison post-test. UT: untreated.

6.4 Discussion

Cytotoxic $CD8^+$ T lymphocytes can be subdivided into different subsets, reminiscent of T helper cell classification[528]. Tc17s display highly suppressed cytotoxic function and express type 17 markers, such as RORγt, IL-23R and IL-17[531]. The polarising conditions required for the differentiation of Tc17 cells, as well as their cytokine profile, are similar to those of $CD4^+$ Th17 lymphocytes[531]. More specifically, naïve $CD8^+$ T cells can be induced to differentiate into an IL-17-producing subset in the presence of IL-6 and TGFβ[531]. IL-6 is sufficient to activate STAT3 and induce the expression of IL-21 and IL-23R in differentiating $CD8^+$ T cells[531]. However, IL-6 is also crucial for the development of cytotoxic potential[545]. The role of TGFβ is therefore to target Eomes and T-bet to suppress IFNγ/granzyme B expression and offset the pro-cytotoxic effects of IL-6[546,547]. Similarly to $CD4^+$ T cells, IL-23 is not critical for Tc17 differentiation: IL-23 alone only slightly induces IL-17A expression in naïve $CD8^+$ T cell cultures[548]. Rather, IL-23 promotes the stabilisation, proliferation and pathogenicity of the $CD8^+$ $IL\text{-}17^+$ population[126,549].

Considering the numerous similarities with regard to their developmental regulation, it stands to reason that mCRAMP may exert similar effects on $CD8^+$ T cells compared to $CD4^+$. I have shown that mCRAMP is a $CD4^+$ Th17 differentiation enhancing factor. I therefore sought to determine whether this host defence peptide exerts similar effects on $CD8^+$ T cells.

6.4.1 mCRAMP enhances Tc17 differentiation

Preliminary data from the laboratory demonstrated that mCRAMP-deficient mice were incapable of producing IL-17 during inflammation induced by HKST (**Chapter 1: Figure 1.10**). $CD8^+$ T cells can produce IL-17 in response to various stimuli and may therefore contribute to the type-17 response induced by HKST[533]. $CD4^+$ T lymphocytes that developed in the absence of mCRAMP did not possess an obvious underlying defect that could be responsible for their inability to produce IL-17 during inflammation *in vivo*. I have shown that this is also true for $CD8^+$ T cells: $CD8^+$ lymphocytes from naïve mCRAMP KO mice displayed relatively normal cytokine responses at resting state and were capable of producing IL-17 following culture

with exogenous cytokines (IL-6, IL-23 and TGFβ), although perhaps to a slightly less extent than WT cells.

6.4.1.1 mCRAMP increases CD8⁺ T cell activation

mCRAMP increased the percentage of activated $CD8^+$ $CD62L^-$ $CD44^+$ T cells, indicating that the peptide increases the activation status of $CD8^+$ T lymphocytes cultured under Tc17-polarizing conditions, similarly to $CD4^+$ T cells.

Furthermore, mCRAMP significantly upregulated the expression of PD1. As discussed previously, PD1 has long been associated with T cell exhaustion[550–552]. For example, Ma et al. demonstrated that $CD8^+$ $PD1^{hi}$ T cells are significantly enriched in hepatocellular carcinoma tumours compared to adjacent non-tumour tissue and highly express exhaustion-related inhibitory markers such as TIM3 and CTLA4[551]. Intrahepatic hepatitis C virus (HCV)-specific $CD8^+$ T cells from patients suffering from chronic HCV infection are also highly PD1 positive and profoundly dysfunctional[552]. However, my results do not suggest that mCRAMP increases $CD8^+$ T cell exhaustion, as they do not display a compromised capacity to produce pro-inflammatory cytokines and appear to proliferate more than their untreated counterparts. Nonetheless, future investigations will include long-term cultures (up to 14 days) in order to assess whether or not mCRAMP does indeed have any effect on T cell exhaustion.

The above findings are in accordance with those published by Petrelli et al., who found that $CD8^+$ $PD1^+$ T lymphocytes that accumulate in the synovial fluid of patients with juvenile idiopathic arthritis are metabolically active effector cells that do not display an exhausted signature[553]. Gene expression profiling revealed that these $CD8^+$ $PD1^+$ T cells were enriched for pathways relating to cell cycle, proliferation, cytotoxicity and pro-inflammatory signalling[553]. The authors suggested that increased PD1 expression is in fact an indicator of functional T cell adaptation to the chronically inflamed milieu[553]. Moreover, Blackburn et al. found that while cytokine production did not strictly correlate with the amount of PD1 expressed by $CD8^+$ T cells, it was tightly linked to their ability to degranulate[554]. Tc17s display suppressed cytotoxic function[531]. It is therefore possible that increased PD1 reflects this and is an indicator of Tc17 potential.

On the other hand, the upregulation of PD1 could represent a mechanism through which mCRAMP reduces the pathogenic potential of $CD8^+$ T cells. Zhang and Xu demonstrated that activated $PDL1^+$ neutrophils inhibit the proliferation of $PD1^+$ T cells and dampen T cell-mediated responses *in vitro*[555]. By increasing the percentage of $CD8^+$ $PD1^+$ T lymphocytes, mCRAMP could consequently render them more vulnerable to suppression.

6.4.1.2 mCRAMP increases the frequency of IL-17-producing $CD8^+$ T lymphocytes

The addition of mCRAMP to Tc17 cultures led to an increase in the expression of the RORγt master regulator. Similarly to during Th17 differentiation, RORγt plays an important role in Tc17 polarisation[531]. For instance, Huber and colleagues observed a significant induction of *Rorc* mRNA in $CD8^+$ T cells cultured in the presence of TGFβ and IL-6[531]. Furthermore, *Rorc* gene expression was increased in $CD8^+$ $IL\text{-}17^+$ T cells in psoriatic skin, unlike $CD8^+$ $IL\text{-}17^-$ T lymphocytes which expressed high levels of *Tbx21* (T-bet)[556]. Mechanistically, RORγt promotes IL-17 production by directly targeting the IL-17 conserved noncoding sequence 2 (CNS-2) enhancer region in $CD8^+$ T cells[557].

However, overexpression of RORγt in $CD8^+$ T lymphocytes under non-polarizing conditions induces only low frequencies of IL-17-producing cells, indicating that RORγt is necessary but not sufficient for IL-17 expression[531]. This is likely due to the antagonising activity of Eomes on RORγt function: levels of this transcription factor negatively correlate with Tc17 development[531]. Several other factors are therefore required to promote the suppression of Eomes and act cooperatively on the *Il17* locus to effectively induce expression of this cytokine[557]. For example, STAT3 and IRF4 work together to function as a molecular switch for cell fate decisions in $CD8^+$ T cells[557]. IRF4-deficient mice display enhanced expression of Eomes and impaired IL-17 production[532].

The increase in RORγt expression following exposure to mCRAMP was accompanied by a concentration-dependent increase in the percentage of $CD8^+$ $IL\text{-}17A^+$ T cells. More specifically, this rose 1.56-fold, which was around half that observed in $CD4^+$ T helper cells (**Chapter 3: Figure 3.8**). There is very little published work that has tried to identify Tc17 enhancing factors. For instance, while the AHR agonist FICZ increases IL-17 production by

$CD4^+$ T cells, no such effect is seen in $CD8^+$ T lymphocytes[558]. Pick and colleagues demonstrated that CTLA4 showcased a cell intrinsic ability to enhance Tc17 differentiation by sustaining the expression of IL-17-inducing factors such as STAT3, RORγt and IRF4[559]. However, to my knowledge, no other direct Tc17 endogenous or exogenous inducing factors have been identified[541].

IL-17A production was significantly increased when culturing sorted $CD8^+$ T cells under Tc17-polarizing conditions with mCRAMP, indicating that similarly to $CD4^+$ T lymphocytes, the peptide can act directly on this cell type. However, the percentages of $CD8^+$ IL-$17A^+$ T cells obtained using pure CD8 cultures were substantially lower than those achieved when using whole single cell splenic suspensions. One possible reason for this is that DCs provide higher quality activation signals than αCD3/CD28 antibodies. $CD8^+$ T lymphocytes may also require additional signals or growth factors that are provided by other cell types present within the spleen. To address this, multiplex cytokine profiling could be performed on supernatants from splenic cultures, similar to that performed by Lehmann et al., to identify potential mediators that could then be added to $CD8^+$ T cells to boost Tc17 differentiation[560].

In steady state, Tc17 cells predominantly produce IL-17F, unlike $CD4^+$ Th17 lymphocytes, which only express IL-17A[398]. This is in accordance with my data, which revealed greater frequencies of $CD8^+$ IL-$17F^+$ T cells in the liver, lungs, spleen, mesenteric lymph nodes and Peyer's patches, compared to $CD8^+$ IL-$17A^+$. In addition, Chang and colleagues detected the presence of IL-17A and IL-17F double-producing $CD8^+$ T cells in bronchoscopic biopsies from patients with chronic obstructive pulmonary disease (COPD), at percentages similar to $CD4^+$ T cells[540]. I found that mCRAMP induced a similar IL-17A/IL-17F expression profile in $CD8^+$ T cells compared to $CD4^+$ when using a TGFβ/IL-6 differentiation protocol, which is potentially due to their similar regulatory requirements[557]. More specifically, mCRAMP increased the percentages of IL-$17F^+$ and IL-$17A^+$ IL-$17F^+$ T cells in Tc17 cultures. However, unlike $CD4^+$ T lymphocytes, mCRAMP also significantly increased the frequency of $CD8^+$ IL-17A single-positive lymphocytes.

mCRAMP upregulated the expression of AHR in $CD4^+$ T cells and acted via this transcription factor to specifically promote the development of $CD4^+$ IL-17A/IL-17F dual-producers

(**Chapter 4: Figures 4.6 & 4.7**). Hayes and colleagues demonstrated that Tc17s express baseline *Ahr* mRNA but do not upregulate its expression during polarisation (unlike $CD4^+$ T lymphocytes), indicating that AHR ligands play a greater role in Th17 development compared to Tc17[541]. My results diverge from this: I found that the frequency of $CD8^+$ AHR^+ T cells increased during polarisation and that mCRAMP increased this further on day 1 (but not on day 2). Potential explanations as to the differences between my results and the Hayes study could include different cells (naïve $CD8^+$ T lymphocytes vs. whole single cell splenic suspensions) and/or polarizing cocktail (Hayes: no IL-23, added IL-1β)[558].

Based on observations made for $CD4^+$ T lymphocytes, I sought to determine what effects an AHR antagonist would have on IL-17 production by $CD8^+$ T cells following exposure to mCRAMP. Similarly to $CD4^+$ T lymphocytes, the addition of CH223191 abolished the increase in the percentage of $CD8^+$ $IL\text{-}17A^+$ $Il\text{-}17F^+$ T cells normally induced by the peptide. Furthermore, the AHR antagonist also prevented an increase in the frequency of $CD8^+$ IL-17A single-positive cells. This was in contrast to $CD4^+$ T lymphocytes, who displayed no significant differences in the frequency of $IL\text{-}17A^+$ $IL\text{-}17F^-$ cells. This highlights potential differences between $CD4^+$ and $CD8^+$ T lymphocytes and suggests that mCRAMP enhances the generation of $CD8^+$ $IL\text{-}17A^+$ $IL\text{-}17F^-$ and IL-17A/IL-17F co-expressers in an AHR-dependent manner.

6.4.1.3 mCRAMP skews cytotoxic T cell differentiation away from the Tc1 lineage

mCRAMP downregulated the expression of genes classically associated with the Th1 lineage in $CD4^+$ T cells cultured under Th17-driving conditions (**Chapter 4: Figure 4.1**). Furthermore, mCRAMP decreased the frequency of $CD4^+$ $T\text{-}bet^+$ and $CD4^+$ $IFN\gamma^+$ T cells in Th17 cultures (**Chapter 4: Figure 4.5**). Similar effects were observed in $CD8^+$ T lymphocytes, suggesting that this host defence peptide also skews $CD8^+$ T cell differentiation away from the Tc1 lineage to promote Tc17 development.

Master regulators, such as T-bet, shape immune responses by activating one genetic program whilst simultaneously silencing the activity of factors that drive the differentiation of other subsets[465,466]. For example, T-bet and Eomes drive Tc1 polarization but have been shown to also suppress alternative $CD8^+$ T cell fates (Tc2 and Tc17)[529,561]. Indeed, Zhu and colleagues

demonstrated that 2 to 3-fold more T-bet-deficient $CD8^+$ T lymphocytes became IL-17 producers when cultured under Tc17-polarizing conditions, compared to WT cells[561]. Tc17s therefore express diminished levels of T-bet and Eomes[126]. To confirm these results, future investigations should explore changes in the expression of Eomes, as this transcription factor is also crucial for the development of Tc1 lymphocytes[529].

Tc1 lymphocytes produce large quantities of IFNγ[528]. mCRAMP significantly decreased the percentage of $CD8^+$ $IFN\gamma^+$ T cells when cultured under Tc17-driving conditions, reflecting the downregulation of T-bet expression and the skewing of cytotoxic T cell differentiation. IFNγ is a well-known suppressor of type-17 immune responses[39]. Similarly to $CD4^+$ T cells, the downregulation of T-bet and subsequent reduction in IFNγ production therefore supports the hypothesis that mCRAMP skews $CD8^+$ T cell differentiation away from the Tc1 lineage and in doing so, also promotes Tc17 development.

Tc17 effector cells are highly plastic and can convert into cytotoxic IFNγ-producing cells that express both RORγt and T-bet[542]. For instance, IL-12 induces the conversion of Tc17 lymphocytes into IL-17/IFNγ dual-producers[542]. Satoh et al. demonstrated that this is driven by the epigenetic suppression of the *Socs3* gene promoter[543]. In the present study, I did not detect any $CD8^+$ $IL\text{-}17A^+$ $IFN\gamma^+$ T cells *in vitro* but a significant proportion of $CD8^+$ T lymphocytes expressed both RORγt and T-bet. Similarly to $CD4^+$ T cells, the addition of mCRAMP decreased their frequency following 3 days of culture under Tc17-driving conditions. $CD8^+$ T cells that co-express RORγt and T-bet are expanded in patients with distal bile duct cancer: they display a hyperactivated TCR signalling signature, which results in enhanced proliferation and a strong pro-inflammatory phenotype but impaired ability to sustain cytotoxic immune responses[544]. The reduced frequency of this cellular subset could indicate that mCRAMP does not increase Tc17 pathogenicity, or at least not by upregulating T-bet and/or IFNγ expression.

6.4.2 Mechanism of action

6.4.2.1 mCRAMP increases the expression of receptors required for Tc17 differentiation

$CD4^+$ T lymphocytes did not upregulate their expression of IL-23R or IL-6R following exposure to mCRAMP (**Chapter 3: Figure 3.3.13**). However, I observed a significant increase in the percentage of $CD8^+$ $IL\text{-}6R^+$ T lymphocytes on day 1 when cultured under Tc17-driving conditions and in the presence of mCRAMP. Signalling via IL-6R induces the activation of STAT3, which promotes the transcription of signature Tc17 genes such as *Rorc*, *Il21* and *Il23r*[531]. Yang et al. found that IL-6R was expressed by naïve ($CD44^{lo}$), but not activated ($CD44^{hi}$), $CD8^+$ T cells, and that the frequency of IL-6R-expressing $CD8^+$ T cells was lower than in $CD4^+$ T lymphocytes[562]. This is in accordance with the data presented here. Furthermore, the authors suggested that the expression of IL-6R defines a subset of $CD8^+$ T cells that are capable of producing IL-21, an important cytokine required for the expansion of Tc17 lymphocytes[562]. The early upregulation of IL-6R by mCRAMP could therefore increase Tc17 potential.

In addition, IL-23R is not expressed by naïve $CD8^+$ T cells and is upregulated following IL-6 induction[556]. Ciric and colleagues demonstrated that IL-23 drives the development of pathogenic IL-17-producing $CD8^+$ T cells[549]. More specifically, Tc17s generated with TGFβ and IL-6 were not diabetogenic, unlike those treated with IL-23, which specifically upregulated IL-17F and potently induced disease[549]. Furthermore, $CD8^+$ $IL\text{-}17^+$ T lymphocytes from EAE mice display elevated levels of IL-23R, which increases their pathogenicity[532]. I have shown that mCRAMP significantly increased the percentage of $CD8^+$ $IL\text{-}23R^+$ T cells on day 2. This could increase the sensitivity of $CD8^+$ T lymphocytes to IL-23 and subsequently promote the generation of a more pathogenic population of IL-17-producing cells.

On the other hand, I have provided evidence suggesting that mCRAMP requires TGFβ to increase RORγt expression and thereby enhance Tc17 differentiation. It is therefore not clear how an increase in the expression of IL-6R and IL-23R could help the peptide achieve this. In the future, it will therefore be necessary to also examine the expression of TGFβR.

Nonetheless, the changes observed in the expression of IL-6R and IL-23R by $CD8^+$ T cells were contrary to those found for $CD4^+$ T lymphocytes, which displayed no significant differences

following exposure to mCRAMP. This supports the hypothesis that mCRAMP exhibits differential effects on $CD4^+$ and $CD8^+$ T cells and suggests that the mechanism it employs to promote Tc17 differentiation differs from that which amplifies Th17 responses. However, it is also possible that, due to high variability, the observations made in the present study are inaccurate. It will therefore be beneficial to repeat the above experiments and analyse the expression of IL-6R, IL-23R and TGFβR by PCR, instead of flow cytometry.

6.4.2.2 mCRAMP is a $CD8^+$ T cell survival factor

mCRAMP increased the viability of the whole $CD8^+$ T cell population when cultured with IL-6, IL-23 and TGFβ, as determined by annexin/PI staining; although, unlike $CD4^+$ T lymphocytes, this was only statistically significant on day 2 and therefore may not be biologically relevant. However, examining the survival of $CD8^+$ T cells (also on day 2) by analysing the uptake of a fixable live/dead stain revealed that mCRAMP significantly decreased the percentages of 'dead' total $CD8^+$, IL-$17A^+$ and IFNγ^+ T lymphocytes. This suggests that mCRAMP is a $CD8^+$ T cell survival factor. These results are in contrast to those published by Mader and colleagues, who demonstrated that human cathelicidin (LL-37) induced granzyme-mediated apoptosis in cytotoxic $CD8^+$ T lymphocytes[378]. However, the concentration of LL-37 used in the Mader study (40 μg/mL) was substantially larger than what I typically add to my cultures (2.5 μM, approx. 10 μg/mL)[378]. It is therefore possible that the observations made by Mader et al. are a result of the cytotoxic side effects of the peptide, which have frequently been noted at high concentrations.

Unlike for $CD4^+$ T helper cells, there appeared to be a small but significant increase in the proliferation of total $CD8^+$ T cells. This could account for enhanced CD8 survival and supports observations made by Mihailovic and colleagues, who demonstrated that the immunization of $ApoE^{-/-}$ mice with synthetic mCRAMP increased the proliferation of cytotoxic $CD8^+$ T cells[563]. However, mCRAMP did not increase the proliferation of $CD8^+$ RORγt^+ T cells, suggesting that there must be another subset that are proliferating more in response to mCRAMP.

TGFβ alone inhibits $CD8^+$ T cell proliferation but this is abrogated in the presence of IL-6, which instead promotes expansion[546]. An increase in proliferation in response to mCRAMP could therefore represent one of the downstream effects of boosting TGFβ signals. However, mCRAMP also decreased the percentage of necrotic $CD8^+$ T cells in the absence of Tc17-stimualting cytokines, indicating once again that there are at least two, different T cell signalling pathways induced by mCRAMP, one of which is non-specific and does not require TGFβ to protect $CD8^+$ T lymphocytes from death.

Liu et al. found that Tc17 cells are more resistant than Tc1s to activation-induced cell death[564]. Furthermore, terminally differentiated or senescent T cells are more prone to apoptosis[565]. Flores-Santibáñez and colleagues demonstrated that Tc17 cells present high levels of the CD73 ectonucleotidase and produce significant quantities of adenosine, which arrests $CD8^+$ T cell differentiation, favours IL-17 production and promotes stem cell-like properties[565]. Tc17 lymphocytes therefore display a less differentiated status and consequently suffer significantly lower apoptosis-induced cell death than Tc1 cells[565]. mCRAMP could therefore increase overall T cell viability simply by skewing cytotoxic T cell differentiation towards the Tc17 lineage.

mCRAMP decreased the percentage of dead $CD8^+$ IL-$17A^+$ T cells but had no significant effect on the proliferation of $CD8^+$ RORγt^+ T lymphocytes, indicating that the peptide does not boost Tc17 differentiation by increasing the proliferative capacity of these subsets. *In vitro*-generated Tc17s present a memory phenotype[566]. Nanjappa et al. observed that memory Tc17 cells display higher levels of basal homeostatic proliferation and were more resistant to apoptosis than Tc1s, despite expressing lower levels of anti-apoptotic molecules such as BCL2 and BCL-XL[567]. Moreover, another study has shown that, unlike naïve $CD8^+$ T cells, memory $CD8^+$ T lymphocytes stimulated in the presence of TGFβ display enhanced survival and increased production of IL-17[568]. One might therefore conclude that, by boosting TGFβ and its downstream effects, mCRAMP increases this memory phenotype, which protects Tc17 cells from death and amplifies Tc17 differentiation.

On the other hand, mCRAMP also significantly increased the viability of $CD8^+$ IFNγ^+ T lymphocytes. This was in contrast to $CD4^+$ IFNγ^+ T helper cells, who were not protected from

death. Interestingly, mCRAMP decreased the proliferation of $CD8^+$ T-bet^+ T lymphocytes in Tc17 cultures. These cells likely represent those producing IFNγ and a reduction in their proliferative capacity may account for, in part, the decrease observed in their frequency but not an increase in their survival. Nonetheless, this highlights a clear difference in the effects of mCRAMP on $CD8^+$ T lymphocytes compared to $CD4^+$ and taken together, suggests that the peptide does not increase Tc17 polarization by simply protecting $CD8^+$ IL-17 producers and/or by promoting the death of non-Tc17 cell types.

6.4.2.3 mCRAMP requires TGFβ to enhance Tc17 differentiation in vitro

Similarly to $CD4^+$ T lymphocytes, mCRAMP significantly upregulated the expression of PD1 by $CD8^+$ T cells when the cells were cultured under non-lineage-driving conditions. However, the peptide had no effect on the percentages of $CD8^+$ IL-17^+ T lymphocytes in the absence of Tc17-polarizing cytokines. This suggests that at least two, separate T cell signalling pathways are induced by mCRAMP, which are likely shared between $CD4^+$ and $CD8^+$ T lymphocytes.

More specifically, my results indicate that the pathway responsible for increasing the development of IL-17-producing $CD8^+$ T cells cultured under Tc17-driving conditions requires IL-6, IL-23 and/or TGFβ. mCRAMP failed to increase RORγt expression by $CD8^+$ T lymphocytes when they were generated in the absence of TGFβ. This supports the hypothesis that, similarly to $CD4^+$ T cells, mCRAMP specifically requires TGFβ to promote Tc17 differentiation. TGFβ helps maintain an IL-17 producing subset by repressing IL-6-induced cytotoxic differentiation via STAT3 and RORγt, which could be enhanced by mCRAMP[557].

However, the contribution of TGFβ to Tc17 skewing may not be as important as for Th17 differentiation, at least *in vivo*. For example, TGFβ neutralisation in mice does not have a considerable effect on the frequency of $CD8^+$ IL-17^+ T cells[569]. Furthermore, transgenic TGFβRIIDN mice that express a dominant negative TGFβ receptor and in which TGFβ signalling is ultimately impaired, cannot produce $CD4^+$ Th17 cells but $CD8^+$ IL-17^+ lymphocytes are still present[570]. The physiological relevance of the effects of mCRAMP on $CD8^+$ Tc17 cells therefore remains unclear.

6.4.3 Are Tc17 cells generated in the presence of mCRAMP more or less pathogenic?

Ciric and colleagues demonstrated that IL-23 promotes the pathogenicity of $CD8^+$ Tc17s by upregulating their expression of IL-17F and not IL-17A[549]. I have shown that mCRAMP significantly increased the frequency of $CD8^+$ IL-17F single-positive cells, as well as IL-17A/IL-17F dual-producers. Unlike $CD4^+$ T cells, mCRAMP may therefore promote the development of a more pathogenic population of Tc17 lymphocytes, which could promote autoimmunity in certain conditions such as multiple sclerosis, where this cytotoxic T cell subset is enriched[532].

Furthermore, mCRAMP significantly increased the frequency of $CD8^+$ IL-17A single-positive T cells. Huber and colleagues demonstrated that IL-17A produced by Tc17 lymphocytes contributes to the initiation of CNS autoimmunity[532]. More specifically, surface-bound IL-17A promoted the generation of $CD4^+$ Th17 cells and rendered them more encephalitogenic during induction of EAE[532]. An increase in the proportion of $CD8^+$ IL-$17A^+$ IL-$17F^-$ T cells could therefore be another indicator of a potentially more pathogenic population of Tc17s.

In addition, increasing the longevity of $CD8^+$ IL-17^+ T cells could result in a prolonged period where they are able to produce inflammatory cytokines, which could in turn lead to greater immunopathology if left unchecked. Frohm et al. reported that human cathelicidin (LL-37) is upregulated in the psoriatic epidermis[284]. Moreover, IL-17-producing $CD8^+$ T cells accumulate in psoriatic skin lesions and have been implicated in driving disease pathology: neutralization of Tc17 lymphocytes effectively prevents psoriasis development *in vivo*[571,572]. Cathelicidin could therefore contribute to disease activity by increasing the survival of pathogenic $CD8^+$ Tc17 cells.

On the other hand, human Tc17s have been shown to harbour potent immune suppressive potential compared to other cytotoxic $CD8^+$ subsets[573]. As a result, Tc17s may play an important regulatory function against effector $CD4^+$ cells. Indeed, acute relapses of multiple sclerosis are characterised by a deficit of $CD8^+$ T cell-mediated immune suppression[574]. It is therefore possible that by enhancing Tc17 differentiation, mCRAMP promotes the development of a less pathogenic and more regulatory subset of $CD8^+$ T cells.

6.5 Summary

To summarize, I have shown that mCRAMP acts directly on $CD8^+$ T lymphocytes *in vitro* to increase their activation status, upregulate RORγt expression and boost Tc17 polarization. My results also indicate that, similarly to $CD4^+$ T helper cells, mCRAMP downregulates T-bet expression and decreases IFNγ production, suggesting that the peptide skews cytotoxic $CD8^+$ T cell differentiation away from the Tc1 lineage. Furthermore, at least two, separate T cell signalling pathways are induced by mCRAMP, one of which requires TGFβ in order to amplify IL-17 production and one which increases PD1 expression even in the absence of polarizing cytokines.

However, I have also provided evidence that suggests mCRAMP exerts differential effects on $CD4^+$ and $CD8^+$ T cells. For example, mCRAMP significantly increases the percentage of $CD8^+$ IL-$17A^+$ IL-$17F^-$ T cells, upregulates their expression of IL-6R and IL-23R and increases the proliferation of the $CD8^+$ T cell population as a whole, none of which was the case for $CD4^+$ T lymphocytes. In addition, I observed several differences in the viability of $CD8^+$ T cells exposed to mCRAMP. More specifically, mCRAMP failed to protect $CD4^+$ IFNγ^+ T helper cells from death but the peptide significantly increased the survival of $CD8^+$ IFNγ^+ Tc17s, despite inducing a decrease in their frequency and impairing the proliferation of T-bet^+ lymphocytes.

CHAPTER 7

Concluding remarks and future directions

I have shown that mouse cathelicidin (mCRAMP) acts directly on $CD4^+$ and $CD8^+$ T lymphocytes to enhance the development of IL-17-producing cells and skew differentiation away from the Th1/Tc1 lineage. Furthermore, I have provided evidence suggesting that mCRAMP induces at least two, different signalling pathways in T lymphocytes. More specifically, mCRAMP required TGFβ to increase RORγt expression. However, mCRAMP also upregulated PD1 and promoted the survival of both $CD4^+$ and $CD8^+$ T cells when they were cultured under non-lineage-driving conditions in the absence of polarizing cytokines.

I have also highlighted a requirement for T cells to sense mCRAMP during the first 24 hours of activation in order to amplify type-17 responses, implying that this interaction takes place in the lymph nodes *in vivo*. In addition, my results suggest that neutrophils are the likely cellular source of this host defence peptide during inflammation induced by heat-killed *S. typhimurium*, a model in which mCRAMP-deficient mice fail to produce IL-17 (**Figure 7.1**).

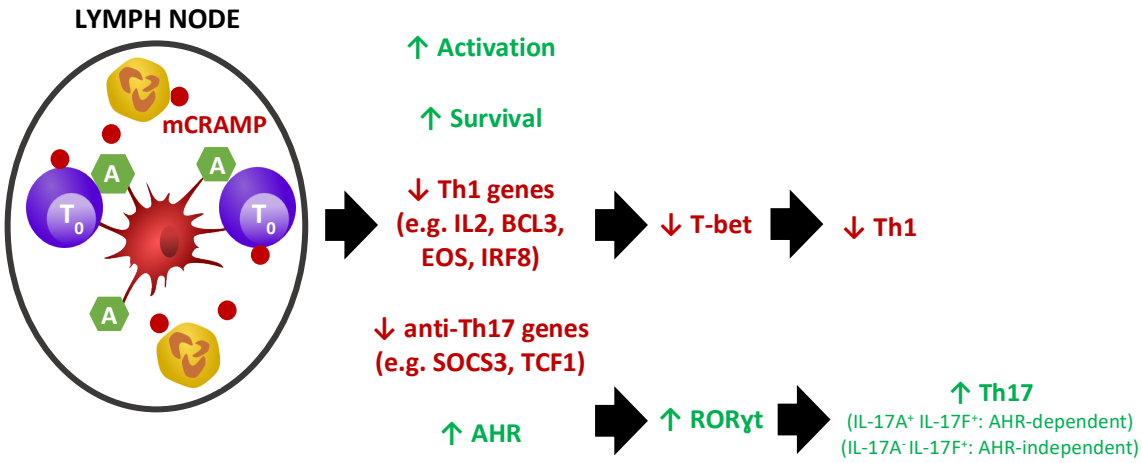


Figure 7.1: mCRAMP is a Th17 differentiation enhancing factor. During inflammation induced by heat-killed *Salmonella typhimurium*, neutrophils are recruited to the draining lymph nodes where they release the host defence peptide, mCRAMP. mCRAMP acts directly on naïve T cells during the first 24 hours of activation to increase their activation status and viability. mCRAMP downregulates the expression of Th1-related and anti-Th17 genes to decrease T-bet and increase RORγt expression, thereby skewing T helper cell differentiation away from the Th1 lineage and towards a Th17 phenotype. mCRAMP specifically promotes the development of IL-17A/IL-17F dual producers in an AHR dependent manner, as well as $CD4^+$ IL-$17A^-$ IL-$17F^+$ T lymphocytes via an AHR-independent pathway. T_o: naïve T cell; A: antigen.

7.1 mCRAMP is a novel Th17 and Tc17 differentiation enhancing factor

In addition to cytokines such as IL-23, which enhances IL-17 production by $CD4^+$ Th17 (and $CD8^+$ Tc17) cells, a number of other exogenous and endogenous Th17 inducers of varying potency have previously been identified. For example, Song et al. demonstrated that vitamin C increased the percentage of $CD4^+$ $IL\text{-}17^+$ T lymphocytes *in vitro* following three days of culture under Th17-driving conditions, although it did not increase RORγt expression or have any effect on IFNγ production (unlike mCRAMP)[575]. Furthermore, as discussed previously, Duarte et al. reported that culturing sorted naïve $CD4^+$ T cells under Th17-driving conditions with AHR agonists, such as FICZ, TCDD, ITE (2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester) and 3-MC (3-methylcholanthrene), increased IL-17 production around 2-fold[474]. Neutrophil elastase has also been shown to promote human Th17 differentiation by processing DC-derived CXCL8 into a truncated, potent Th17-inducing form[234].

However, there is very little published work that has tried to identify $CD8^+$ Tc17 enhancing factors. For instance, while AHR agonists increase IL-17 production by $CD4^+$ T lymphocytes, Hayes and colleagues demonstrated that $CD8^+$ Tc17 cells were largely refractory to exogenous FICZ[541]. They suggested that this was because Tc17s express baseline *Ahr* mRNA but do not upregulate its expression during polarisation (unlike $CD4^+$ T lymphocytes), indicating that AHR ligands and the activation of the AHR pathway may play a greater role in Th17 development compared to Tc17[541].

I have shown that mCRAMP significantly increased the frequency of $CD4^+$ $IL\text{-}17^+$ T cells in Th17 cultures. The fold change in the number of IL-17-producing $CD4^+$ T lymphocytes (6.5-fold in sorted $CD4^+$ T cells; 1.99-fold in splenic cultures) suggests that mCRAMP is of equal or superior potency compared to previously identified Th17 enhancers. Moreover, mCRAMP also amplified the differentiation of $CD8^+$ Tc17 cells. mCRAMP therefore represents a novel, potent, locally produced Th17 and Tc17 differentiation enhancing factor that contributes to the amplification of type-17 responses during inflammation.

7.2 mCRAMP is a T cell survival factor

The present study has uncovered several novel findings. Cathelicidin has been shown to suppress apoptosis of several cell types, including cardiomyocytes, endothelial cells, keratinocytes and dermal fibroblasts[358,414,415,576]. I have shown that mCRAMP increases $CD4^+$ and $CD8^+$ T cell viability, which is contrary to published work by Mader et al., who demonstrated that human cathelicidin (LL-37) induces granzyme-mediated apoptosis of regulatory and $CD8^+$ cytotoxic T lymphocytes[359,360]. To my knowledge, this is the first demonstration of a neutrophil peptide increasing the survival of T cells.

Interestingly, mCRAMP protected $CD4^+$ $IL\text{-}17A^+$ T cells from death, but not those that produced IFNγ. The fact that this host defence peptide appears to protect one T helper cell subset from death over another is quite unusual, although several factors have been shown to selectively promote apoptosis of specific $CD4^+$ T lymphocytes. For example, galectin-1 functions as a Th2 cytokine that preferentially induces Th1 apoptosis[577]. Similarly, IL-10 controls Th2 cell survival by upregulating granzyme B expression during allergic airway inflammation[578]. On the other hand, Elyaman and colleagues suggested that IL-9 could act as a survival factor for regulatory T lymphocytes, as this cytokine rescued Tregs from apoptotic cell death *in vitro*[579].

These differences in survival were not a result of changes in the proliferative capacity of $CD4^+$ $ROR\gamma t^+$ or $T\text{-}bet^+$ T cells. This is similar to findings by Coccia et al., who demonstrated a key role for IL-1β and IL-1R in the survival of pathogenic Th17 cells in the colon: the authors showed that reduced accumulation of naïve $CD4^+$ T lymphocytes from IL-1R-deficient mice following adoptive transfer to $RAG^{-/-}$ animals was not caused by impaired proliferation or migration of these cells; instead, the authors suggested that IL-1β promoted Th17 survival by upregulating the expression of anti-apoptotic proteins such as BCL2 and BCL-XL[580].

7.3 The potential importance of mCRAMP-induced IL-2 suppression

mCRAMP significantly decreased the expression of IL-2 in $CD4^+$ T lymphocytes cultured under Th17-driving conditions. This was confirmed by measuring the concentration of IL-2 in cell

culture supernatants, which showed an almost complete suppression of production following exposure to mCRAMP.

IL-2 is a potent Th17 antagonist and I have highlighted multiple pathways that are potentially modulated by mCRAMP, which regulate the inhibitory effects of this cytokine[442]. For example, mCRAMP requires TGFβ to increase RORγt expression and has previously been shown to suppress IL-2[462]. In addition, mCRAMP upregulates the aryl hydrocarbon receptor (AHR), which cooperates with STAT3 to induce the expression of Aiolos; Aiolos binds to the *Il2* promoter and induces chromatin modifications that result in IL-2 silencing[478]. mCRAMP also downregulates the expression of EOS, which is required for IL-2 production by $CD4^+$ T cells[461]. Due to the multitude of pathways influenced by mCRAMP which affect IL-2 production, one might speculate that the downregulation of this cytokine is one of the key mechanisms through which it acts to enhance Th17 (and Tc17) differentiation. It would therefore be interesting to determine whether the addition of recombinant IL-2 to Th17 cultures abolishes the increase in IL-17-producing T lymphocytes normally induced in response to mCRAMP.

IL-2 is a key factor that drives the clonal proliferation of activated T lymphocytes[581]. One might therefore predict that the suppression of IL-2 production by mCRAMP would result in reduced T cell proliferation. For example, this could account for a decrease in the number of T-bet/IFNγ-expressing cells. However, this was not the case. Nonetheless, neutrophils are prominent producers of mCRAMP and have been shown to strongly inhibit T cell proliferation under certain conditions[249,582]. One might therefore speculate that the release of neutrophil-derived cathelicidin and the subsequent suppression of IL-2, is one of the mechanisms by which neutrophils potentiate T cell responses.

In addition, IL-2 is a recognised T cell survival factor: Kelly et al. demonstrated that this cytokine activates the protein kinase AKT, which protects T lymphocytes from apoptosis following growth factor withdrawal[583]. However, I have shown that despite the fact that mCRAMP downregulated IL-2 expression by $CD4^+$ T cells cultured under Th17-driving conditions, it also increased their viability. This could be explained, in part, by the fact that Th17 lymphocytes are significant producers of IL-21, which has been shown to be a critical

regulator of $CD4^+$ (and $CD8^+$) T cell survival during priming under IL-2 deprivation conditions[584].

7.4 Differential effects of mCRAMP on $CD4^+$ T helper and $CD8^+$cytotoxic T cells

I have shown that mCRAMP enhances both $CD4^+$ Th17 and $CD8^+$ Tc17 differentiation. However, several differences were observed between the two cell types. For example, mCRAMP had no effect on the percentage of IL-17A single-positive $CD4^+$ T cells in IL-17-inducing cultures but significantly increased the frequency of $CD8^+$ IL-$17A^+$ IL-$17F^-$ T lymphocytes (in an AHR-dependent manner). This suggests that mCRAMP exerts differential effects on $CD4^+$ and $CD8^+$ T cells.

Furthermore, mCRAMP increased the proportions of $CD8^+$ IL-$6R^+$ and IL-$23R^+$ T lymphocytes but had no effect on the expression of these receptors in $CD4^+$ T cells. This could indicate that $CD8^+$ T cells cultured under Tc17-driving conditions are more sensitive to IL-6 and IL-23. However, this does not explain the requirement for TGFβ and analysing these receptors by flow cytometry may not provide the most reliable readout: the quality of staining was highly variable and must therefore be confirmed by PCR.

Differences were also observed in the proliferative capacity of $CD4^+$ and $CD8^+$ T cells following exposure to mCRAMP: $CD8^+$ T lymphocytes treated with mCRAMP appeared to proliferate more than their untreated counterparts, whereas the peptide did not have any significant effect on the proliferation of $CD4^+$ T cells. However, it is important to note that mCRAMP did not increase the proliferation of $CD8^+$ RORγt$^+$ T cells (and decreased that of $CD8^+$ T-bet$^+$ T lymphocytes), which suggests that the peptide exerts effects on another Tc subset.

I have shown that mCRAMP skews both $CD4^+$ and $CD8^+$ T cell differentiation away from the Th1/Tc1 lineage, demonstrated by a significant decrease in T-bet expression and IFNγ production. mCRAMP increased the viability of $CD4^+$ IL-17^+ T cells but did not decrease the percentage of dead $CD4^+$ IFNγ^+ T lymphocytes. This lack of protection could be responsible for the decrease in the frequency of $CD4^+$ IFNγ^+ T cells, which is normally seen in Th17 cultures

following exposure to mCRAMP. Conversely, while $CD8^+$ $IL\text{-}17^+$ T lymphocytes were also protected from death, so too were $CD8^+$ $IFN\gamma^+$ T cells. T-bet-expressing $CD8^+$ T cells that had been cultured in the presence of mCRAMP exhibited impaired proliferation compared to their untreated counterparts. This suggests that mCRAMP skews cytotoxic $CD8^+$ T cell differentiation away from the Tc1 lineage by suppressing their proliferation (as opposed to failing to protect them from cell death).

7.5 Neutrophils, cathelicidin and type-17 responses

There is a limited amount of published data suggesting that neutrophils can promote the differentiation of IL-17-producing T lymphocytes. For example, Thewissen et al. found that neutrophils dose-dependently increase IL-17 production by $CD4^+$ T cells in an *in vitro*-co-culture model[492]. In addition, neutrophils have been shown to assist in the induction of Th17-specific responses during vaccination against *Mycobacterium tuberculosis*[493]. Neutrophil cytoplasts, the remnants of NETs following expulsion of their nuclear DNA, also induce Th17 differentiation in severe asthma[233]. However, the exact mechanisms through which they achieve this remain unknown.

I have speculated that neutrophils are the cellular source of mCRAMP, which amplifies Th17 and Tc17 differentiation following release in the lymph node during inflammation induced by HKST. My results suggest that mCRAMP requires $TGF\beta$ in order to enhance the development of IL-17-producing $CD4^+$ and $CD8^+$ T lymphocytes. Both mouse and human neutrophils can produce $TGF\beta$[195,242]. It is therefore possible that neutrophils play a dual role in driving Th17 differentiation by simultaneously releasing mCRAMP and producing Th17-polarizing cytokines.

Furthermore, I have highlighted a potentially novel role for mCRAMP in promoting the survival of T lymphocytes. Neutrophils increase the survival of B cells and plasma cells via their expression of BAFF and APRIL, as well as preventing NK cells from undergoing activation-induced cell death in tumours[585–587]. However, there is little evidence suggesting that neutrophils protect T lymphocytes from apoptosis, although the inverse has been shown: production of GM-CSF by Th17 cells enhances neutrophil survival by increasing the

expression of anti-apoptotic BCL2 and preventing caspase activation[588]. My results therefore point towards an additional unique pathway by which neutrophils can modulate the development of the adaptive T cell response with some sophistication.

7.6 Future directions

There are several avenues of research that can be pursued to further explore the role of mCRAMP in the development of Th17 and Tc17 responses. For example, the IL-17-inducing differentiation protocol used throughout this study (IL-6, IL-23 and TGFβ) has been shown to preferentially promote the expression of IL-17F[398]. I have shown that mCRAMP significantly increased the proportions of IL-17F single-positive and IL-17A/IL-17F co-expressing $CD4^+$ T lymphocytes in a TGFβ-dependent manner. Wanke et al. demonstrated that culturing $CD4^+$ T lymphocytes with IL-1β leads to the development of predominately IL-17A-expressing cells[398]. It would therefore be interesting to establish whether mCRAMP exhibits similar effects on $CD4^+$ (and $CD8^+$) T cells when stimulated with IL-1β. This would help clarify whether the effects of mCRAMP described in this study are simply a by-product of the cell culture conditions used.

Furthermore, it remains to be seen whether $CD4^+$ Th17 and $CD8^+$ Tc17 cells generated in the presence of mCRAMP *in vitro* are more or less pathogenic than those cultured without. My Nanostring data indicated that the expression of certain effector molecules, such as IL-2, CXCR3 and IL-22, which are known to identify a population of damaging, pro-inflammatory lymphocytes, was either downregulated or unaffected by mCRAMP[479]. Moreover, the upregulation of immunomodulatory genes, such as *Ahr* and *Maf*, suggest that they possess a more regulatory phenotype. To address this, one could perform a T cell transfer model of colitis, in which T cells cultured under IL-17-inducing conditions, with or without mCRAMP, are injected intraperitoneally into immunodeficient $RAG^{-/-}$ mice[406]. If $IL\text{-}17^+$ lymphocytes generated in the presence of mCRAMP are indeed less pathogenic, I would hypothesise that animals receiving mCRAMP-treated cells would display delayed onset of colitis and potentially lower disease scores than controls.

mCRAMP also increased the expression of the aryl hydrocarbon receptor (AHR) in $CD4^+$ T cells in Th17 cultures. The addition of an AHR antagonist abolished the increase in the percentage of $CD4^+$ $IL\text{-}17A^+$ $IL\text{-}17F^+$ T cells but had no effect on the increase in IL-17F single-positive lymphocytes. This suggests that there are at least two pathways induced by mCRAMP, one of which is AHR-dependent and one which is AHR-independent. To further explore this possibility, single-cell transcriptome and signalling pathway analysis could be used to dissect the differences between IL17A and IL-17F single- or double-producing lymphocytes following exposure to mCRAMP.

Finally, I have suggested that it is unlikely that mCRAMP is an endogenous AHR ligand due to the fact that other small, structurally different peptides (e.g. hBD2) also enhance Th17 differentiation. However, further studies can be undertaken to confirm this. For example, bioinformatic and molecular modelling approaches could be employed to establish whether mCRAMP and/or LL-37 are capable of potentially interacting with the AHR ligand binding domain based on their sequence and structure.

7.7 Targeting cathelicidin as a therapy for autoimmune disease

To conclude, I have shown that mouse cathelicidin (mCRAMP) is a novel Th17 and Tc17 differentiation enhancing factor and suggested that neutrophils may be the cellular source of this peptide *in vivo* during inflammation induced by HKST. IL-17-producing T cells have been heavily implicated in driving the pathogenesis of numerous chronic, pro-inflammatory and autoimmune disorders, including rheumatoid arthritis (RA) and psoriasis[589]. The presence of neutrophils and increased levels of cathelicidin have been correlated with many of these diseases. For example, neutrophils migrate to the articular cavity during the early stages of RA, where they become activated and prone to NETosis[495]. Elevated levels of LL-37, as well as IL-17, have been detected in the synovial fluid of RA patients[365,590]. Moreover, the abundant presence of neutrophils in psoriatic lesions serves as a typical histopathologic hallmark of disease[591]. Frohm et al. found that LL-37 is increased in the psoriatic epidermis, which acts as an autoantigen to induce Th17 and Tc17 activation[284,334]. Taken together, one might therefore suggest that cathelicidin could be considered as a potential therapeutic target.

However, cathelicidin is a host defence peptide that possesses anti-bacterial, anti-viral and anti-fungal activity, as well as immunomodulatory properties[277,285]. Blocking its function may well dampen the pathogenic, autoimmune type-17 response, however in doing so, patients could be rendered more susceptible to opportunistic infection. For example, individuals with morbus Kostmann syndrome, whose neutrophils are deficient for LL-37, are vulnerable to recurrent infections and often present with severe periodontal disease[311]. On the other hand, one could argue that the host defence system has evolved families of peptides and proteins with overlapping functions, which could be indicative of biological redundancy[592,593]. Other antimicrobial peptides, such as the defensins, could therefore potentially take over such a role in the absence of cathelicidin. Morbus Kostmann neutrophils display reduced concentrations of α-defensins, which could explain why this redundant protection is not observed in patients with this form of severe congenital neutropenia[311].

Conversely, using cathelicidin as a treatment for microbial infections may have unexpected consequences, such as increased Th17/Tc17 differentiation and uncontrolled IL-17 production, which could lead to autoimmune inflammation. Specificity of targeting would therefore be of paramount importance to avoid any unintended side effects.

Another important question that remains to be answered is whether or not blocking the amplification of Th17/Tc17 development by cathelicidin would ameliorate or worsen disease. I have shown that mCRAMP increases IL-17F production and the expression of immunoregulatory genes such as *Ahr* in $CD4^+$ T cells cultured under Th17-driving conditions *in vitro*, which suggests that they may be less pathogenic than their untreated counterparts. It is therefore possible that suppressing the IL-17-boosting effects of cathelicidin could render these T cell subsets more pro-inflammatory and subsequently more damaging.

References

1. Chaplin, D. D. Overview of the immune response. *J. Allergy Clin. Immunol.* (2010). doi:10.1016/j.jaci.2009.12.980
2. Buchmann, K. Evolution of innate immunity: Clues from invertebrates via fish to mammals. *Front. Immunol.* (2014). doi:10.3389/fimmu.2014.00459
3. Riera Romo, M., Pérez-Martínez, D. & Castillo Ferrer, C. Innate immunity in vertebrates: An overview. *Immunology* (2016). doi:10.1111/imm.12597
4. Mogensen, T. H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews* (2009). doi:10.1128/CMR.00046-08
5. Flajnik, M. F. & Kasahara, M. Origin and evolution of the adaptive immune system: Genetic events and selective pressures. *Nature Reviews Genetics* (2010). doi:10.1038/nrg2703
6. Smith, K. A. Toward a molecular understanding of adaptive immunity: A chronology, part I. *Frontiers in Immunology* (2012). doi:10.3389/fimmu.2012.00369
7. Krangel, M. S. Mechanics of T cell receptor gene rearrangement. *Current Opinion in Immunology* (2009). doi:10.1016/j.coi.2009.03.009
8. Pennock, N. D. *et al.* T cell responses: naive to memory and everything in between. *AJP Adv. Physiol. Educ.* **37**, 273–283 (2013).
9. Famili, F., Wiekmeijer, A. S. & Staal, F. J. The development of T cells from stem cells in mice and humans. *Future Science OA* (2017). doi:10.4155/fsoa-2016-0095
10. Chao, D. L., Davenport, M. P., Forrest, S. & Perelson, A. S. The effects of thymic selection on the range of T cell cross-reactivity. *Eur. J. Immunol.* (2005). doi:10.1002/eji.200535098
11. Hunter, M. C., Teijeira, A. & Halin, C. T cell trafficking through lymphatic vessels. *Frontiers in Immunology* (2016). doi:10.3389/fimmu.2016.00613
12. Cose, S., Brammer, C., Khanna, K. M., Masopust, D. & Lefrançois, L. Evidence that a significant number of naive T cells enter non-lymphoid organs as part of a normal migratory pathway. *Eur. J. Immunol.* (2006). doi:10.1002/eji.200535539
13. Chen, W. & Zhu, C. Mechanical regulation of T-cell functions. *Immunol. Rev.* (2013). doi:10.1111/imr.12122
14. Sansom, D. M. CD28, CTLA-4 and their ligands: Who does what and to whom? *Immunology* (2000). doi:10.1046/j.1365-2567.2000.00121.x
15. Croft, M. Costimulation of T cells by OX40, 4-1BB, and CD27. *Cytokine and Growth Factor Reviews* (2003). doi:10.1016/S1359-6101(03)00025-X
16. Rothoeft, T., Gonschorek, A., Bartz, H., Anhenn, O. & Schauer, U. Antigen dose, type of antigen-presenting cell and time of differentiation contribute to the T helper 1/T helper 2 polarization of naive T cells. *Immunology* (2003). doi:10.1111/j.1365-2567.2003.01758.x
17. Pozzi, L.-A. M., Maciaszek, J. W. & Rock, K. L. Both Dendritic Cells and Macrophages Can Stimulate Naive CD8 T Cells In Vivo to Proliferate, Develop Effector Function, and Differentiate into Memory Cells. *J. Immunol.* (2005). doi:10.4049/jimmunol.175.4.2071
18. Kara, E. E. *et al.* Tailored Immune Responses: Novel Effector Helper T Cell Subsets in Protective Immunity. *PLoS Pathogens* (2014). doi:10.1371/journal.ppat.1003905
19. Halle, S., Halle, O. & Förster, R. Mechanisms and Dynamics of T Cell-Mediated Cytotoxicity In Vivo. *Trends in Immunology* (2017). doi:10.1016/j.it.2017.04.002

20. Calzascia, T. *et al.* TNF-α is critical for antitumor but not antiviral T cell immunity in mice. *J. Clin. Invest.* (2007). doi:10.1172/JCI32567
21. Ruby, J. & Ramshaw, I. The antiviral activity of immune CD8+ T cells is dependent on interferon- γ. *Lymphokine Cytokine Res.* (1991).
22. Lopez, J. A. *et al.* Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack. *Blood* (2013). doi:10.1182/blood-2012-07-446146
23. Volpe, E., Sambucci, M., Battistini, L. & Borsellino, G. Fas-fas ligand: Checkpoint of t cell functions in multiple sclerosis. *Frontiers in Immunology* (2016). doi:10.3389/fimmu.2016.00382
24. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* (1986).
25. Zhu, J., Yamane, H. & Paul, W. E. Differentiation of Effector CD4 T Cell Populations. *Annu. Rev. Immunol.* (2010). doi:10.1146/annurev-immunol-030409-101212
26. Luckheeram, R. V., Zhou, R., Verma, A. D. & Xia, B. CD4 +T cells: Differentiation and functions. *Clinical and Developmental Immunology* (2012). doi:10.1155/2012/925135
27. Hsieh, C. S. *et al.* Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science (80-.).* (1993). doi:10.1126/science.8097338
28. Afkarian, M. *et al.* T-bet is a STATI-induced regulator for IL-12R expression in naïve CD4+ T cells. *Nat. Immunol.* (2002). doi:10.1038/ni794
29. Thierfelder, W. E. *et al.* Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* (1996). doi:10.1038/382171a0
30. Usui, T. *et al.* T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. *J. Exp. Med.* (2006). doi:10.1084/jem.20052165
31. Suzuki, Y., Orellana, M. A., Schreiber, R. D. & Remington, J. S. Interferon-γ: The major mediator of resistance against Toxoplasma gondii. *Science (80-.).* (1988). doi:10.1126/science.3128869
32. Swain, S. L., Weinberg, A. D., English, M. & Huston, G. IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* (1990).
33. Zhu, J., Guo, L., Watson, C. J., Hu-Li, J. & Paul, W. E. Stat6 Is Necessary and Sufficient for IL-4's Role in Th2 Differentiation and Cell Expansion. *J. Immunol.* (2001). doi:10.4049/jimmunol.166.12.7276
34. Zhu, J. *et al.* Conditional deletion of Gata3 shows its essential function in TH1-TH2 responses. *Nat. Immunol.* (2004). doi:10.1038/ni1128
35. Usui, T., Nishikomori, R., Kitani, A. & Strober, W. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rβ2 chain or T-bet. *Immunity* (2003). doi:10.1016/S1074-7613(03)00057-8
36. Horiuchi, S. *et al.* Genome-Wide Analysis Reveals Unique Regulation of Transcription of Th2-Specific Genes by GATA3. *J. Immunol.* (2011). doi:10.4049/jimmunol.1100179
37. Punnonen, J., Yssel, H. & De Vries, J. E. The relative contribution of IL-4 and IL-13 to human IgE synthesis induced by activated CD4+ or CD8+ T cells. *J. Allergy Clin. Immunol.* (1997). doi:10.1016/S0091-6749(97)70276-8
38. Warringa, R. A. *et al.* Modulation of eosinophil chemotaxis by interleukin-5. *Am. J. Respir. Cell Mol. Biol.* (1992). doi:10.1165/ajrcmb/7.6.631
39. Park, H. *et al.* A distinct lineage of CD4 T cells regulates tissue inflammation by

producing interleukin 17. *Nat. Immunol.* (2005). doi:10.1038/ni1261
40. Yang, J., Sundrud, M. S., Skepner, J. & Yamagata, T. Targeting Th17 cells in autoimmune diseases. *Trends in Pharmacological Sciences* (2014). doi:10.1016/j.tips.2014.07.006
41. Kimura, A., Naka, T. & Kishimoto, T. IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. *Proc. Natl. Acad. Sci. U. S. A.* (2007). doi:10.1073/pnas.0705268104
42. Yang, X. O. *et al.* STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J. Biol. Chem.* (2007). doi:10.1074/jbc.C600321200
43. Ivanov, I. I. *et al.* The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+T Helper Cells. *Cell* (2006). doi:10.1016/j.cell.2006.07.035
44. Burgler, S. *et al.* Differentiation and functional analysis of human TH17 cells. *J. Allergy Clin. Immunol.* (2009). doi:10.1016/j.jaci.2008.12.017
45. Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* (1999). doi:10.1084/jem.190.7.995
46. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* (1995).
47. Read, S., Malmström, V. & Powrie, F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25+CD4+ regulatory cells that control intestinal inflammation. *J. Exp. Med.* (2000). doi:10.1084/jem.192.2.295
48. Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *J. Immunol.* (2003). doi:10.1038/ni904
49. Chen, W. J. *et al.* Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3. *J. Exp. Med.* (2003). doi:10.1084/jem.20030152
50. Davidson, T. S., DiPaolo, R. J., Andersson, J. & Shevach, E. M. Cutting Edge: IL-2 Is Essential for TGF-β-Mediated Induction of Foxp3 + T Regulatory Cells . *J. Immunol.* (2007). doi:10.4049/jimmunol.178.7.4022
51. Kaplan, M. H., Hufford, M. M. & Olson, M. R. The development and in vivo function of T helper 9 cells. *Nature Reviews Immunology* (2015). doi:10.1038/nri3824
52. Veldhoen, M. *et al.* Transforming growth factor-β 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* (2008). doi:10.1038/ni.1659
53. Trifari, S., Kaplan, C. D., Tran, E. H., Crellin, N. K. & Spits, H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from TH-17, TH1 and TH2 cells. *Nat. Immunol.* (2009). doi:10.1038/ni.1770
54. Eyerich, S. *et al.* Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J. Clin. Invest.* (2009). doi:10.1172/JCI40202
55. Breitfeld, D. *et al.* Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* (2000). doi:10.1084/jem.192.11.1545
56. Nurieva, R. I. *et al.* Bcl6 mediates the development of T follicular helper cells. *Science (80-.).* (2009). doi:10.1126/science.1176676
57. De Beaucoudtey, L. *et al.* Mutations in STAT3 and IL12RB1 impair the development

of human IL-17-producing T cells. *J. Exp. Med.* (2008). doi:10.1084/jem.20080321
58. Liu, X., Lee, Y. S., Yu, C.-R. & Egwuagu, C. E. Loss of STAT3 in CD4 + T Cells Prevents Development of Experimental Autoimmune Diseases . *J. Immunol.* (2008). doi:10.4049/jimmunol.180.9.6070
59. Harris, T. J. *et al.* Cutting Edge: An In Vivo Requirement for STAT3 Signaling in TH17 Development and TH17-Dependent Autoimmunity. *J. Immunol.* (2007). doi:10.1093/pch/5.5.262
60. Chen, Z. *et al.* Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc. Natl. Acad. Sci. U. S. A.* (2006). doi:10.1073/pnas.0600666103
61. Crome, S. Q., Wang, A. Y., Kang, C. Y. & Levings, M. K. The role of retinoic acid-related orphan receptor variant 2 and IL-17 in the development and function of human CD4+ T cells. *Eur. J. Immunol.* (2009). doi:10.1002/eji.200838908
62. Yang, X. O. *et al.* T Helper 17 Lineage Differentiation Is Programmed by Orphan Nuclear Receptors RORα and RORγ. *Immunity* (2008). doi:10.1016/j.immuni.2007.11.016
63. Yoshida, Y. *et al.* The Transcription Factor IRF8 Activates Integrin-Mediated TGF-β Signaling and Promotes Neuroinflammation. *Immunity* (2014). doi:10.1016/j.immuni.2013.11.022
64. Veldhoen, M., Hocking, R. J., Flavell, R. A. & Stockinger, B. Signals mediated by transforming growth factor-β initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. *Nat. Immunol.* (2006). doi:10.1038/ni1391
65. Gorelik, L., Constant, S. & Flavell, R. A. Mechanism of transforming growth factor β-induced inhibition of T helper type 1 differentiation. *J. Exp. Med.* (2002). doi:10.1084/jem.20012076
66. Qin, H. *et al.* TGF-β Promotes Th17 Cell Development through Inhibition of SOCS3. *J. Immunol.* (2009). doi:10.4049/jimmunol.0801986
67. Kimura, A. & Kishimoto, T. IL-6: Regulator of Treg/Th17 balance. *European Journal of Immunology* (2010). doi:10.1002/eji.201040391
68. Liu, H. & Rohowsky-Kochan, C. Regulation of IL-17 in Human CCR6 + Effector Memory T Cells . *J. Immunol.* (2008). doi:10.4049/jimmunol.180.12.7948
69. Chen, Z., Tato, C. M., Muul, L., Laurence, A. & O'Shea, J. J. Distinct regulation of interleukin-17 in human T helper lymphocytes. *Arthritis Rheum.* (2007). doi:10.1002/art.22866
70. Acosta-Rodriguez, E. V., Napolitani, G., Lanzavecchia, A. & Sallusto, F. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat. Immunol.* (2007). doi:10.1038/ni1496
71. Manel, N., Unutmaz, D. & Littman, D. R. The differentiation of human TH-17 cells requires transforming growth factor-β and induction of the nuclear receptor RORγt. *Nat. Immunol.* (2008). doi:10.1038/ni.1610
72. Nurieva, R. *et al.* Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* (2007). doi:10.1038/nature05969
73. Awasthi, A. & Kuchroo, V. K. Th17 cells: From precursors to players in inflammation and infection. *International Immunology* (2009). doi:10.1093/intimm/dxp021
74. Sonderegger, I., Kisielow, J., Meier, R., King, C. & Kopf, M. IL-21 and IL-21R are not required for development of Th17 cells and autoimmunity in vivo. *Eur. J. Immunol.* (2008). doi:10.1002/eji.200838511

75. Zhou, L. *et al.* IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* (2007). doi:10.1038/ni1488
76. Jain, R. *et al.* Interleukin-23-Induced Transcription Factor Blimp-1 Promotes Pathogenicity of T Helper 17 Cells. *Immunity* (2016). doi:10.1016/j.immuni.2015.11.009
77. McGeachy, M. J. *et al.* The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat. Immunol.* (2009). doi:10.1038/ni.1698
78. Sun, M. *et al.* RORγt Represses IL-10 Production in Th17 Cells To Maintain Their Pathogenicity in Inducing Intestinal Inflammation. *J. Immunol.* (2019). doi:10.4049/jimmunol.1701697
79. Veldhoen, M. *et al.* The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* (2008). doi:10.1038/nature06881
80. Veldhoen, M., Hirota, K., Christensen, J., O'Garra, A. & Stockinger, B. Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. *J. Exp. Med.* (2009). doi:10.1084/jem.20081438
81. Kimura, A., Naka, T., Nohara, K., Fujii-Kuriyama, Y. & Kishimoto, T. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proc. Natl. Acad. Sci.* (2008). doi:10.1073/pnas.0804231105
82. Schraml, B. U. *et al.* The AP-1 transcription factor Batf controls T H 17 differentiation. *Nature* (2009). doi:10.1038/nature08114
83. Huber, M. *et al.* IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proc. Natl. Acad. Sci. U. S. A.* (2008). doi:10.1073/pnas.0809077106
84. Mudter, J. *et al.* IRF4 regulates IL-17A promoter activity and controls RORγt-dependent Th17 colitis in vivo. *Inflamm. Bowel Dis.* (2011). doi:10.1002/ibd.21476
85. Krausgruber, T. *et al.* T-bet is a key modulator of IL-23-driven pathogenic CD4 + T cell responses in the intestine. *Nat. Commun.* (2016). doi:10.1038/ncomms11627
86. Diveu, C. *et al.* IL-27 Blocks RORc Expression to Inhibit Lineage Commitment of Th17 Cells. *J. Immunol.* (2009). doi:10.4049/jimmunol.0801162
87. El-behi, M. *et al.* Differential Effect of IL-27 on Developing versus Committed Th17 Cells. *J. Immunol.* (2009). doi:10.4049/jimmunol.0900735
88. Yang, X. *et al.* Opposing regulation of the Il17 locus through direct, reciprocal actions of STAT3 and STAT5. *Nat. Immunol.* (2011). doi:10.1038/ni.1995.Opposing
89. Guenova, E. *et al.* IL-4 abrogates TH17 cell-mediated inflammation by selective silencing of IL-23 in antigen-presenting cells. *Proc. Natl. Acad. Sci. U. S. A.* (2015). doi:10.1073/pnas.1416922112
90. Ivanov, I. I. *et al.* Specific Microbiota Direct the Differentiation of IL-17-Producing T-Helper Cells in the Mucosa of the Small Intestine. *Cell Host Microbe* (2008). doi:10.1016/j.chom.2008.09.009
91. Ivanov, I. I. *et al.* Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. *Cell* (2009). doi:10.1016/j.cell.2009.09.033
92. Stockinger, B. & Omenetti, S. The dichotomous nature of T helper 17 cells. *Nature Reviews Immunology* (2017). doi:10.1038/nri.2017.50
93. Sano, T. *et al.* An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses. *Cell* (2015). doi:10.1016/j.cell.2015.08.061
94. Garidou, L. *et al.* The Gut Microbiota Regulates Intestinal CD4 T Cells Expressing

RORγt and Controls Metabolic Disease. *Cell Metab.* (2015). doi:10.1016/j.cmet.2015.06.001
95. Wu, H. J. *et al.* Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* (2010). doi:10.1016/j.immuni.2010.06.001
96. Chewning, J. H. & Weaver, C. T. Development and Survival of Th17 Cells within the Intestines: The Influence of Microbiome- and Diet-Derived Signals. *J. Immunol.* (2014). doi:10.4049/jimmunol.1401835
97. Atarashi, K. *et al.* ATP drives lamina propria TH17 cell differentiation. *Nature* (2008). doi:10.1038/nature07240
98. McGeachy, M. J., Cua, D. J. & Gaffen, S. L. The IL-17 Family of Cytokines in Health and Disease. *Immunity* (2019). doi:10.1016/j.immuni.2019.03.021
99. Chang, S. H. & Dong, C. A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses. *Cell Res.* (2007). doi:10.1038/cr.2007.35
100. Gaffen, S. L. Structure and signalling in the IL-17 receptor family. *Nature Reviews Immunology* (2009). doi:10.1038/nri2586
101. Zrioual, S. *et al.* IL-17RA and IL-17RC Receptors Are Essential for IL-17A-Induced ELR + CXC Chemokine Expression in Synoviocytes and Are Overexpressed in Rheumatoid Blood . *J. Immunol.* (2008). doi:10.4049/jimmunol.180.1.655
102. Laan, M. *et al.* Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J. Immunol.* (1999).
103. Liu, R. *et al.* IL-17 Promotes Neutrophil-Mediated Immunity by Activating Microvascular Pericytes and Not Endothelium. *J. Immunol.* (2016). doi:10.4049/jimmunol.1600138
104. Fossiez, F. *et al.* T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J. Exp. Med.* (1996). doi:10.1084/jem.183.6.2593
105. Schwarzenberger, P. *et al.* Requirement of Endogenous Stem Cell Factor and Granulocyte-Colony-Stimulating Factor for IL-17-Mediated Granulopoiesis. *J. Immunol.* (2000). doi:10.4049/jimmunol.164.9.4783
106. Ye, P. *et al.* Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J. Exp. Med.* (2001). doi:10.1084/jem.194.4.519
107. Liang, S. C. *et al.* An IL-17F/A Heterodimer Protein Is Produced by Mouse Th17 Cells and Induces Airway Neutrophil Recruitment. *J. Immunol.* (2007). doi:10.4049/jimmunol.179.11.7791
108. Liang, S. C. *et al.* Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med.* (2006). doi:10.1084/jem.20061308
109. El-Behi, M. *et al.* The encephalitogenicity of TH 17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat. Immunol.* (2011). doi:10.1038/ni.2031
110. Sonderegger, I. *et al.* GM-CSF mediates autoimmunity by enhancing IL-6-dependent Th17 cell development and survival. *J. Exp. Med.* (2008). doi:10.1084/jem.20071119
111. Yeste, A. *et al.* IL-21 induces IL-22 production in CD4+ T cells. *Nat. Commun.* (2014). doi:10.1038/ncomms4753
112. Rutz, S. *et al.* Transcription factor c-Maf mediates the TGF-β-dependent suppression of IL-22 production in T H17 cells. *Nat. Immunol.* (2011). doi:10.1038/ni.2134
113. Perusina Lanfranca, M., Lin, Y., Fang, J., Zou, W. & Frankel, T. Biological and

pathological activities of interleukin-22. *Journal of Molecular Medicine* (2016). doi:10.1007/s00109-016-1391-6

114. Radaeva, S., Sun, R., Pan, H. N., Hong, F. & Gao, B. Interleukin 22 (IL-22) Plays a Protective Role in T Cell-mediated Murine Hepatitis: IL-22 Is a Survival Factor for Hepatocytes via STAT3 Activation. *Hepatology* (2004). doi:10.1002/hep.20184
115. Aujla, S. J. *et al.* IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat. Med.* (2008). doi:10.1038/nm1710
116. Zheng, Y. *et al.* Interleukin-22, a TH17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* (2007). doi:10.1038/nature05505
117. Tesmer, L. A., Lundy, S. K., Sarkar, S. & Fox, D. A. Th17 cells in human disease. *Immunological Reviews* (2008). doi:10.1111/j.1600-065X.2008.00628.x
118. Happel, K. I. *et al.* Cutting Edge: Roles of Toll-Like Receptor 4 and IL-23 in IL-17 Expression in Response to Klebsiella pneumoniae Infection . *J. Immunol.* (2003). doi:10.4049/jimmunol.170.9.4432
119. Lu, Y. J. *et al.* Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog.* (2008). doi:10.1371/journal.ppat.1000159
120. Khader, S. A. *et al.* IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat. Immunol.* (2007). doi:10.1038/ni1449
121. Chung, D. R. *et al.* CD4 + T Cells Mediate Abscess Formation in Intra-abdominal Sepsis by an IL-17-Dependent Mechanism . *J. Immunol.* (2003). doi:10.4049/jimmunol.170.4.1958
122. Martinez, N. E. *et al.* Regulatory T cells and Th17 cells in viral infections: Implications for multiple sclerosis and myocarditis. *Future Virology* (2012). doi:10.2217/fvl.12.44
123. Bermejo-Martin, J. F. *et al.* Th1 and Th17 hypercytokinemia as early host response signature in severe pandemic influenza. *Crit. Care* (2009). doi:10.1186/cc8208
124. Hou, W., Kang, H. S. & Kim, B. S. Th17 cells enhance viral persistence and inhibit T cell cytotoxicity in a model of chronic virus infection. *J. Exp. Med.* (2009). doi:10.1084/jem.20082030
125. Yang, W. *et al.* Interferon-gamma negatively regulates Th17-mediated immunopathology during mouse hepatitis virus infection. *J. Mol. Med.* (2011). doi:10.1007/s00109-010-0711-5
126. Hamada, H. *et al.* Tc17, a Unique Subset of CD8 T Cells That Can Protect against Lethal Influenza Challenge. *J. Immunol.* (2009). doi:10.4049/jimmunol.0801814
127. Mengesha, B. G. & Conti, H. R. The role of IL-17 in protection against mucosal Candida infections. *Journal of Fungi* (2017). doi:10.3390/jof3040052
128. Puel, A. *et al.* Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science (80-.).* (2011). doi:10.1126/science.1200439
129. Conti, H. R. *et al.* Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J. Exp. Med.* (2009). doi:10.1084/jem.20081463
130. Mansouri, M., Mansouri, P., Raze, A. A. & Jadali, Z. The potential role of Th17 lymphocytes in patients with psoriasis. *An. Bras. Dermatol.* (2018). doi:10.1590/abd1806-4841.20186123
131. Ha, H. L. *et al.* IL-17 drives psoriatic inflammation via distinct, target cell-specific mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* (2014). doi:10.1073/pnas.1400513111
132. Boniface, K. *et al.* IL-22 Inhibits Epidermal Differentiation and Induces Proinflammatory Gene Expression and Migration of Human Keratinocytes. *J.*

Immunol. (2005). doi:10.4049/jimmunol.174.6.3695
133. Wohn, C. *et al.* Langerinneg conventional dendritic cells produce IL-23 to drive psoriatic plaque formation in mice. *Proc. Natl. Acad. Sci. U. S. A.* (2013). doi:10.1073/pnas.1307569110
134. Nograles, K. E. *et al.* Th17 cytokines interleukin (IL)-17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways. *Br. J. Dermatol.* (2008). doi:10.1111/j.1365-2133.2008.08769.x
135. Chiricozzi, A. *et al.* Integrative responses to IL-17 and TNF-α in human keratinocytes account for key inflammatory pathogenic circuits in psoriasis. *J. Invest. Dermatol.* (2011). doi:10.1038/jid.2010.340
136. Gaffen, S. L. The role of interleukin-17 in the pathogenesis of rheumatoid arthritis. *Current Rheumatology Reports* (2009). doi:10.1007/s11926-009-0052-y
137. Nakae, S., Nambu, A., Sudo, K. & Iwakura, Y. Suppression of Immune Induction of Collagen-Induced Arthritis in IL-17-Deficient Mice. *J. Immunol.* (2003). doi:10.4049/jimmunol.171.11.6173
138. Gullick, N. J. *et al.* Enhanced and persistent levels of interleukin (IL)-17+CD4+ T cells and serum IL-17 in patients with early inflammatory arthritis. *Clin. Exp. Immunol.* (2013). doi:10.1111/cei.12167
139. Cai, L. *et al.* Pathways by which interleukin 17 induces articular cartilage breakdown in vitro and in vivo. *Cytokine* (2001). doi:10.1006/cyto.2001.0939
140. Shahrara, S., Pickens, S. R., Dorfleutner, A. & Pope, R. M. IL-17 Induces Monocyte Migration in Rheumatoid Arthritis. *J. Immunol.* (2009). doi:10.4049/jimmunol.0802246
141. Lubberts, E. *et al.* IL-1-Independent Role of IL-17 in Synovial Inflammation and Joint Destruction During Collagen-Induced Arthritis. *J. Immunol.* (2001). doi:10.4049/jimmunol.167.2.1004
142. Katz, Y., Nadiv, O. & Beer, Y. Interleukin-17 Enhances Tumor Necrosis Factor α-Induced Synthesis of Interleukins 1, 6, and 8 in Skin and Synovial Fibroblasts: A Possible Role as a 'Fine-Tuning Cytokine' in Inflammation Processes. *Arthritis Rheum.* (2001). doi:10.1002/1529-0131(200109)44:9<2176::AID-ART371>3.0.CO;2-4
143. Dos Passos, G. R., Sato, D. K., Becker, J. & Fujihara, K. Th17 Cells Pathways in Multiple Sclerosis and Neuromyelitis Optica Spectrum Disorders: Pathophysiological and Therapeutic Implications. *Mediators of Inflammation* (2016). doi:10.1155/2016/5314541
144. Komiyama, Y. *et al.* IL-17 Plays an Important Role in the Development of Experimental Autoimmune Encephalomyelitis. *J. Immunol.* (2006). doi:10.4049/jimmunol.177.1.566
145. Hedegaard, C. J. *et al.* T helper cell type 1 (Th1), Th2 and Th17 responses to myelin basic protein and disease activity in multiple sclerosis. *Immunology* (2008). doi:10.1111/j.1365-2567.2008.02837.x
146. Jadidi-Niaragh, F. & Mirshafiey, A. Th17 Cell, the new player of neuroinflammatory process in multiple sclerosis. *Scandinavian Journal of Immunology* (2011). doi:10.1111/j.1365-3083.2011.02536.x
147. Huppert, J. *et al.* Cellular mechanisms of IL-17-induced blood-brain barrier disruption. *FASEB J.* (2010). doi:10.1096/fj.09-141978
148. Gálvez, J. Role of Th17 Cells in the Pathogenesis of Human IBD. *ISRN Inflamm.* (2014). doi:10.1155/2014/928461
149. Fujino, S. *et al.* Increased expression of interleukin 17 in inflammatory bowel

disease. *Gut* (2003). doi:10.1136/gut.52.1.65
150. Raza, A. & Shata, M. T. Letter: Pathogenicity of Th17 cells may differ in ulcerative colitis compared with Crohn's disease. *Alimentary Pharmacology and Therapeutics* (2012). doi:10.1111/j.1365-2036.2012.05124.x
151. Hata, K. *et al.* IL-17 stimulates inflammatory responses via NF-κB and MAP kinase pathways in human colonic myofibroblasts. *Am. J. Physiol. - Gastrointest. Liver Physiol.* (2002). doi:10.1152/ajpgi.00494.2001
152. Zhang, Z., Zheng, M., Bindas, J., Schwarzenberger, P. & Kolls, J. K. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflamm. Bowel Dis.* (2006). doi:10.1097/01.MIB.0000218764.06959.91
153. Ogawa, A., Andoh, A., Araki, Y., Bamba, T. & Fujiyama, Y. Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. *Clin. Immunol.* (2004). doi:10.1016/j.clim.2003.09.013
154. Zenewicz, L. A. *et al.* Innate and Adaptive Interleukin-22 Protects Mice from Inflammatory Bowel Disease. *Immunity* (2008). doi:10.1016/j.immuni.2008.11.003
155. Rosales, C. Neutrophil: A cell with many roles in inflammation or several cell types? *Frontiers in Physiology* (2018). doi:10.3389/fphys.2018.00113
156. Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology* (2013). doi:10.1038/nri3399
157. Pillay, J. *et al.* In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood* (2010). doi:10.1182/blood-2010-01-259028
158. Tofts, P. S., Chevassut, T., Cutajar, M., Dowell, N. G. & Peters, A. M. Doubts concerning the recently reported human neutrophil lifespan of 5.4 days. *Blood* (2011). doi:10.1182/blood-2010-10-310532
159. Lawrence, S. M., Corriden, R. & Nizet, V. The Ontogeny of a Neutrophil: Mechanisms of Granulopoiesis and Homeostasis. *Microbiol. Mol. Biol. Rev.* (2018). doi:10.1128/mmbr.00057-17
160. Hidalgo, A., Chilvers, E. R., Summers, C. & Koenderman, L. The Neutrophil Life Cycle. *Trends in Immunology* (2019). doi:10.1016/j.it.2019.04.013
161. Borregaard, N., Sørensen, O. E. & Theilgaard-Mönch, K. Neutrophil granules: a library of innate immunity proteins. *Trends Immunol.* (2007). doi:10.1016/j.it.2007.06.002
162. Le Cabec, V., Cowland, J. B., Calafat, J. & Borregaard, N. Targeting of proteins to granule subsets is determined by timing and not by sorting: The specific granule protein NGAL is localized to azurophil granules when expressed in HL-60 cells. *Proc. Natl. Acad. Sci. U. S. A.* (1996). doi:10.1073/pnas.93.13.6454
163. Sheshachalam, A., Srivastava, N., Mitchell, T., Lacy, P. & Eitzen, G. Granule protein processing and regulated secretion in neutrophils. *Frontiers in Immunology* (2014). doi:10.3389/fimmu.2014.00448
164. Eash, K. J., Greenbaum, A. M., Gopalan, P. K. & Link, D. C. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J. Clin. Invest.* (2010). doi:10.1172/JCI41649
165. De Filippo, K. & Rankin, S. M. CXCR4, the master regulator of neutrophil trafficking in homeostasis and disease. *European Journal of Clinical Investigation* (2018). doi:10.1111/eci.12949
166. Eash, K. J., Means, J. M., White, D. W. & Link, D. C. CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood* (2009). doi:10.1182/blood-2008-09-177287

167. Brackett, C. M., Muhitch, J. B., Evans, S. S. & Gollnick, S. O. IL-17 Promotes Neutrophil Entry into Tumor-Draining Lymph Nodes following Induction of Sterile Inflammation. *J. Immunol.* (2013). doi:10.4049/jimmunol.1103621
168. Hampton, H. R., Bailey, J., Tomura, M., Brink, R. & Chtanova, T. Microbe-dependent lymphatic migration of neutrophils modulates lymphocyte proliferation in lymph nodes. *Nat. Commun.* **6**, 1–11 (2015).
169. Beauvillain, C. *et al.* CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood* (2011). doi:10.1182/blood-2009-11-254490
170. MORITA, E., SCHRÖDER, J. -M & CHRISTOPHERS, E. Differential Sensitivities of Purified Human Eosinophils and Neutrophils to Defined Chemotaxins. *Scand. J. Immunol.* (1989). doi:10.1111/j.1365-3083.1989.tb01175.x
171. Dorward, D. A. *et al.* The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation. *American Journal of Pathology* (2015). doi:10.1016/j.ajpath.2015.01.020
172. Oyoshi, M. K. *et al.* Leukotriene B4-Driven Neutrophil Recruitment to the Skin Is Essential for Allergic Skin Inflammation. *Immunity* (2012). doi:10.1016/j.immuni.2012.06.018
173. Kuijpers, T. W., Hakkert, B. C., Hart, M. H. L. & Roos, D. Neutrophil migration across monolayers of cytokine-prestimulated endothelial cells: A role for platelet-activating factor and IL-8. *J. Cell Biol.* (1992). doi:10.1083/jcb.117.3.565
174. Gay, J. C. Mechanism and regulation of neutrophil priming by platelet-activating factor. *J. Cell. Physiol.* (1993). doi:10.1002/jcp.1041560125
175. Denk, S. *et al.* Complement C5a-Induced Changes in Neutrophil Morphology During Inflammation. *Scand. J. Immunol.* (2017). doi:10.1111/sji.12580
176. Sadik, C. D., Miyabe, Y., Sezin, T. & Luster, A. D. The critical role of C5a as an initiator of neutrophil-mediated autoimmune inflammation of the joint and skin. *Seminars in Immunology* (2018). doi:10.1016/j.smim.2018.03.002
177. Teng, T. S., Ji, A. L., Ji, X. Y. & Li, Y. Z. Neutrophils and immunity: From bactericidal action to being conquered. *Journal of Immunology Research* (2017). doi:10.1155/2017/9671604
178. Leliefeld, P. H. C., Koenderman, L. & Pillay, J. How neutrophils shape adaptive immune responses. *Front. Immunol.* **6**, 1–8 (2015).
179. Rosales, C. & Uribe-Querol, E. Phagocytosis: A Fundamental Process in Immunity. *BioMed Research International* (2017). doi:10.1155/2017/9042851
180. Cabiscol, E., Tamarit, J. & Ros, J. Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int. Microbiol.* (2000). doi:10.2436/im.v3i1.9235
181. Cassatella, M. A., Östberg, N. K., Tamassia, N. & Soehnlein, O. Biological Roles of Neutrophil-Derived Granule Proteins and Cytokines. *Trends in Immunology* (2019). doi:10.1016/j.it.2019.05.003
182. Sengeløv, H. *et al.* Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. *J. Immunol.* (1995).
183. Lei, J. *et al.* The antimicrobial peptides and their potential clinical applications. *American Journal of Translational Research* (2019).
184. Johnson, J. L. *et al.* Interleukin-6 augments neutrophil cytotoxic potential via selective enhancement of elastase release. in *Journal of Surgical Research* (1998). doi:10.1006/jsre.1998.5295
185. Remijsen, Q. *et al.* Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death and Differentiation* (2011). doi:10.1038/cdd.2011.1

186. Brinkmann, V. *et al.* Neutrophil Extracellular Traps Kill Bacteria. *Science (80-.).* (2004). doi:10.1126/science.1092385
187. Papayannopoulos, V. Neutrophil extracellular traps in immunity and disease. *Nature Reviews Immunology* (2018). doi:10.1038/nri.2017.105
188. Lande, R. *et al.* Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci. Transl. Med.* (2011). doi:10.1126/scitranslmed.3001180
189. Perobelli, S. M. *et al.* Plasticity of neutrophils reveals modulatory capacity. *Brazilian J. Med. Biol. Res.* (2015). doi:10.1590/1414-431X20154524
190. Kalyan, S. & Kabelitz, D. When neutrophils meet T cells: Beginnings of a tumultuous relationship with underappreciated potential. *Eur. J. Immunol.* **44**, 627–633 (2014).
191. Ellis, T. N. & Beaman, B. L. Interferon-γ activation of polymorphonuclear neutrophil function. *Immunology* (2004). doi:10.1111/j.1365-2567.2004.01849.x
192. Pelletier, M., Micheletti, A. & Cassatella, M. A. Modulation of human neutrophil survival and antigen expression by activated CD4 + and CD8 + T cells . *J. Leukoc. Biol.* (2010). doi:10.1189/jlb.0310172
193. Van Gisbergen, K. P. J. M., Sanchez-Hernandez, M., Geijtenbeek, T. B. H. & Van Kooyk, Y. Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *J. Exp. Med.* (2005). doi:10.1084/jem.20041276
194. Scapini, P. *et al.* The neutrophil as a cellular source of chemokines. *Immunological Reviews* (2000). doi:10.1034/j.1600-065X.2000.17706.x
195. Kamenyeva, O. *et al.* Neutrophil Recruitment to Lymph Nodes Limits Local Humoral Response to Staphylococcus aureus. *PLoS Pathog.* (2015). doi:10.1371/journal.ppat.1004827
196. Fox, S., Leitch, A. E., Duffin, R., Haslett, C. & Rossi, A. G. Neutrophil apoptosis: Relevance to the innate immune response and inflammatory disease. *Journal of Innate Immunity* (2010). doi:10.1159/000284367
197. Scannell, M. *et al.* Annexin-1 and Peptide Derivatives Are Released by Apoptotic Cells and Stimulate Phagocytosis of Apoptotic Neutrophils by Macrophages. *J. Immunol.* (2007). doi:10.4049/jimmunol.178.7.4595
198. Huynh, M. L. N., Fadok, V. A. & Henson, P. M. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-β1 secretion and the resolution of inflammation. *J. Clin. Invest.* (2002). doi:10.1172/JCI0211638
199. Li, H.-N. *et al.* Secondary necrosis of apoptotic neutrophils induced by the human cathelicidin LL-37 is not proinflammatory to phagocytosing macrophages. *J. Leukoc. Biol.* (2009). doi:10.1189/jlb.0209050
200. Clark, R. & Kupper, T. Old meets new: The interaction between innate and adaptive immunity. *Journal of Investigative Dermatology* (2005). doi:10.1111/j.0022-202X.2005.23856.x
201. Rumble, J. M. *et al.* Neutrophil-related factors as biomarkers in EAE and MS. *J. Exp. Med.* **212**, 23–35 (2015).
202. Lim, K. *et al.* Neutrophil trails guide influenzaspecific CD8+ T cells in the airways. *Science (80-.).* (2015). doi:10.1126/science.aaa4352
203. Russell, C. D., Unger, S. A., Walton, M. & Schwarze, J. The human immune response to respiratory syncytial virus infection. *Clinical Microbiology Reviews* (2017). doi:10.1128/CMR.00090-16
204. Kalyan, S. & Kabelitz, D. When neutrophils meet T cells: Beginnings of a tumultuous

relationship with underappreciated potential. *European Journal of Immunology* (2014). doi:10.1002/eji.201344195

205. Maletto, B. A. *et al.* Presence of neutrophil-bearing antigen in lymphoid organs of immune mice. *Blood* **108**, 3094–3102 (2006).
206. Abadie, V. *et al.* Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood* (2005). doi:10.1182/blood-2005-03-1281
207. Beauvillain, C. *et al.* CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood* **117**, 1196–1204 (2011).
208. Costa, S., Bevilacqua, D., Cassatella, M. A. & Scapini, P. Recent advances on the crosstalk between neutrophils and B or T lymphocytes. *Immunology* (2019). doi:10.1111/imm.13005
209. Hülsdünker, J. *et al.* Neutrophils provide cellular communication between ileum and mesenteric lymph nodes at graft-versus-host disease onset. *Blood* (2018). doi:10.1182/blood-2017-10-812891
210. Abdallah, D. S. A., Egan, C. E., Butcher, B. A. & Denkers, E. Y. Mouse neutrophils are professional antigen-presenting cells programmed to instruct T h1 and T h17 T-cell differentiation. *Int. Immunol.* **23**, 317–326 (2011).
211. Vono, M. *et al.* Neutrophils acquire the capacity for antigen presentation to memory CD4+ T cells in vitro and ex vivo. *Blood* **129**, 1991–2001 (2017).
212. Fanger, N. A. *et al.* Activation of human T cells by major histocompatability complex class II expressing neutrophils: Proliferation in the presence of superantigen, but not tetanus toxoid. *Blood* (1997). doi:10.1182/blood.v89.11.4128
213. Polak, D. *et al.* A novel role for neutrophils in IgE-mediated allergy: Evidence for antigen presentation in late-phase reactions. *J. Allergy Clin. Immunol.* (2019). doi:10.1016/j.jaci.2018.06.005
214. Beauvillain, C. *et al.* Neutrophils efficiently cross-prime naive T cells in vivo. *Blood* (2007). doi:10.1182/blood-2006-12-063826
215. Hufford, M. M. *et al.* Influenza-Infected Neutrophils within the Infected Lungs Act as Antigen Presenting Cells for Anti-Viral CD8+ T Cells. *PLoS One* (2012). doi:10.1371/journal.pone.0046581
216. Schuster, S., Hurrell, B. & Tacchini-Cottier, F. Crosstalk between neutrophils and dendritic cells: a context-dependent process. *J. Leukoc. Biol.* (2013). doi:10.1189/jlb.1012540
217. Bennouna, S., Bliss, S. K., Curiel, T. J. & Denkers, E. Y. Cross-Talk in the Innate Immune System: Neutrophils Instruct Recruitment and Activation of Dendritic Cells during Microbial Infection. *J. Immunol.* (2003). doi:10.4049/jimmunol.171.11.6052
218. Charmoy, M. *et al.* Neutrophil-derived CCL3 is essential for the rapid recruitment of dendritic cells to the site of Leishmania major inoculation in resistant mice. *PLoS Pathog.* (2010). doi:10.1371/journal.ppat.1000755
219. Blomgran, R. & Ernst, J. D. Lung Neutrophils Facilitate Activation of Naive Antigen-Specific CD4 + T Cells during Mycobacterium tuberculosis Infection . *J. Immunol.* (2011). doi:10.4049/jimmunol.1100001
220. Weber, F. C. *et al.* Neutrophils are required for both the sensitization and elicitation phase of contact hypersensitivity. *J. Exp. Med.* (2015). doi:10.1084/jem.20130062
221. Wittamer, V. *et al.* Neutrophil-Mediated Maturation of Chemerin: A Link between Innate and Adaptive Immunity. *J. Immunol.* (2005). doi:10.4049/jimmunol.175.1.487
222. Park, S. J., Burdick, M. D. & Mehrad, B. Neutrophils mediate maturation and efflux of

lung dendritic cells in response to Aspergillus fumigatus germ tubes. *Infect. Immun.* (2012). doi:10.1128/IAI.00097-12

223. Steinbach, K., Piedavent, M., Bauer, S., Neumann, J. T. & Friese, M. A. Neutrophils Amplify Autoimmune Central Nervous System Infiltrates by Maturing Local APCs. *J. Immunol.* **191**, 4531–4539 (2013).
224. Megiovanni, A. M. *et al.* Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *J. Leukoc. Biol.* (2006). doi:10.1189/jlb.0905526
225. Tillack, K., Breiden, P., Martin, R. & Sospedra, M. T Lymphocyte Priming by Neutrophil Extracellular Traps Links Innate and Adaptive Immune Responses. *J. Immunol.* (2012). doi:10.4049/jimmunol.1103414
226. Davey, M. S. *et al.* Human neutrophil clearance of bacterial pathogens triggers anti-microbial γδ T cell responses in early infection. *PLoS Pathog.* (2011). doi:10.1371/journal.ppat.1002040
227. Coeshott, C. *et al.* Converting enzyme-independent release of tumor necrosis factor α and IL-1β from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc. Natl. Acad. Sci. U. S. A.* (1999). doi:10.1073/pnas.96.11.6261
228. Ben-Sasson, S. Z. *et al.* IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc. Natl. Acad. Sci. U. S. A.* (2009). doi:10.1073/pnas.0902745106
229. Minns, D., Smith, K. J. & Findlay, E. G. Orchestration of Adaptive T Cell Responses by Neutrophil Granule Contents. *Mediators of Inflammation* (2019). doi:10.1155/2019/8968943
230. Toussaint, M. *et al.* Host DNA released by NETosis promotes rhinovirus-induced type-2 allergic asthma exacerbation. *Nat. Med.* (2017). doi:10.1038/nm.4332
231. McFarlane, E. *et al.* Neutrophils contribute to development of a protective immune response during onset of infection with Leishmania donovani. *Infect. Immun.* (2008). doi:10.1128/IAI.01388-07
232. Lin, J. *et al.* Neonatal neutrophils stimulated by group B Streptococcus induce a proinflammatory T-helper cell bias. *Pediatr. Res.* (2018). doi:10.1038/pr.2017.272
233. Krishnamoorthy, N. *et al.* Neutrophil cytoplasts induce TH17 differentiation and skew inflammation toward neutrophilia in severe asthma. *Sci. Immunol.* (2018). doi:10.1126/sciimmunol.aao4747
234. Souwer, Y. *et al.* Human TH17 cell development requires processing of dendritic cell–derived CXCL8 by neutrophil elastase. *J. Allergy Clin. Immunol.* (2018). doi:10.1016/j.jaci.2018.01.003
235. Tate, M. D., Brooks, A. G., Reading, P. C. & Mintern, J. D. Neutrophils sustain effective CD8 T-cell responses in the respiratory tract following influenza infection. *Immunol. Cell Biol.* (2012). doi:10.1038/icb.2011.26
236. Tak, T. *et al.* Neutrophil-mediated suppression of influenza-induced pathology requires CD11b/CD18 (MAC-1). *American Journal of Respiratory Cell and Molecular Biology* (2018). doi:10.1165/rcmb.2017-0021OC
237. Mora-Cartín, R. *et al.* Neutrophils dampen adaptive immunity in Brucellosis. *Infect. Immun.* (2019). doi:10.1128/IAI.00118-19
238. Wozniak, K. L., Kolls, J. K. & Wormley, F. L. Depletion of neutrophils in a protective model of pulmonary cryptococcosis results in increased IL-17A production by gamma/delta T cells. *BMC Immunol.* (2012). doi:10.1186/1471-2172-13-65

239. Kasten, K. R., Muenzer, J. T. & Caldwell, C. C. Neutrophils are significant producers of IL-10 during sepsis. *Biochem. Biophys. Res. Commun.* (2010). doi:10.1016/j.bbrc.2010.01.066
240. Ng, T. H. S. *et al.* Regulation of adaptive immunity; the role of interleukin-10. *Frontiers in Immunology* (2013). doi:10.3389/fimmu.2013.00129
241. Doz, E., Lombard, R., Carreras, F., Buzoni-Gatel, D. & Winter, N. Mycobacteria-Infected Dendritic Cells Attract Neutrophils That Produce IL-10 and Specifically Shut Down Th17 CD4 T Cells through Their IL-10 Receptor. *J. Immunol.* (2013). doi:10.4049/jimmunol.1300527
242. Grotendorst, G. R., Smale, G. & Pencev, D. Production of transforming growth factor beta by human peripheral blood monocytes and neutrophils. *J. Cell. Physiol.* (1989). doi:10.1002/jcp.1041400226
243. Pham, C. T. N. Neutrophil serine proteases: Specific regulators of inflammation. *Nature Reviews Immunology* (2006). doi:10.1038/nri1841
244. Bank, U. *et al.* Selective proteolytic cleavage of IL-2 receptor and IL-6 receptor ligand binding chains by neutrophil-derived serine proteases at foci of inflammation. *J. Interf. Cytokine Res.* (1999). doi:10.1089/107999099312957
245. Kessenbrock, K., Dau, T. & Jenne, D. E. Tailor-made inflammation: How neutrophil serine proteases modulate the inflammatory response. *Journal of Molecular Medicine* (2011). doi:10.1007/s00109-010-0677-3
246. Rao, R. M. *et al.* Elastase release by transmigrating neutrophils deactivates endothelial-bound SDF-1α and attenuates subsequent T lymphocyte transendothelial migration. *J. Exp. Med.* (2004). doi:10.1084/jem.20040499
247. Scuderi, P., Nez, P. A., Duerr, M. L., Wong, B. J. & Valdez, C. M. Cathepsin-G and leukocyte elastase inactivate human tumor necrosis factor and lymphotoxin. *Cell. Immunol.* (1991). doi:10.1016/0008-8749(91)90275-G
248. Bank, U., Kuüpper, B. & Ansorge, S. Inactivation of interleukin-6 by neutrophil proteases at sites of inflammation: Protective effects of soluble IL-6 receptor chains. *Adv. Exp. Med. Biol.* (2000).
249. Pillay, J. *et al.* A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J. Clin. Invest.* (2012). doi:10.1172/JCI57990
250. Cemerski, S., Cantagrel, A., Van Meerwijk, J. P. M. & Romagnoli, P. Reactive oxygen species differentially affect T cell receptor-signaling pathways. *J. Biol. Chem.* (2002). doi:10.1074/jbc.M111451200
251. Gelderman, K. A., Hultqvist, M., Holmberg, J., Olofsson, P. & Holmdahl, R. T cell surface redox levels determine T cell reactivity and arthritis susceptibility. *Proc. Natl. Acad. Sci. U. S. A.* (2006). doi:10.1073/pnas.0604571103
252. Schmielau, J. & Finn, O. J. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. *Cancer Res.* (2001).
253. Mensurado, S. *et al.* Tumor-associated neutrophils suppress pro-tumoral IL-17+ γδ T cells through induction of oxidative stress. *PLoS Biol.* (2018). doi:10.1371/journal.pbio.2004990
254. Belikov, A. V., Schraven, B. & Simeoni, L. T cells and reactive oxygen species. *Journal of Biomedical Science* (2015). doi:10.1186/s12929-015-0194-3
255. Rodriguez, P. C., Zea, A. H., Culotta, K. S., Zabaleta, J. & Ochoa Augusto C Ochoa, J. B. Regulation of T cell receptor CD3ζ chain expression by L-arginine. *J. Biol. Chem.* (2002). doi:10.1074/jbc.M110675200

256. Sippel, T. R. *et al.* Arginase I release from activated neutrophils induces peripheral immunosuppression in a murine model of stroke. *J. Cereb. Blood Flow Metab.* (2015). doi:10.1038/jcbfm.2015.103
257. Ingersoll, S. A. *et al.* Mature Cystic Fibrosis Airway Neutrophils Suppress T Cell Function: Evidence for a Role of Arginase 1 but Not Programmed Death-Ligand 1. *J. Immunol.* (2015). doi:10.4049/jimmunol.1500312
258. Rodriguez, P. C., Quiceno, D. G. & Ochoa, A. C. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood* (2007). doi:10.1182/blood-2006-06-031856
259. Feldmeyer, N. *et al.* Arginine deficiency leads to impaired cofilin dephosphorylation in activated human T lymphocytes. *Int. Immunol.* (2012). doi:10.1093/intimm/dxs004
260. Kropf, P. *et al.* Arginase activity mediates reversible T cell hyporesponsiveness in human pregnancy. *Eur. J. Immunol.* (2007). doi:10.1002/eji.200636542
261. Yu, H. R. *et al.* L-Arginine modulates neonatal lymphocyte proliferation through an interleukin-2 independent pathway. *Immunology* (2014). doi:10.1111/imm.12300
262. Jin, H.-T., Ahmed, R. & Okazaki, T. Role of PD-1 in Regulating T-Cell Immunity. in (2010). doi:10.1007/82_2010_116
263. Wang, J. F. *et al.* Up-regulation of programmed cell death 1 ligand 1 on neutrophils may be involved in sepsis-induced immunosuppression: An animal study and a prospective case-control study. *Anesthesiology* (2015). doi:10.1097/ALN.0000000000000525
264. Buddhisa, S., Rinchai, D., Ato, M., Bancroft, G. J. & Lertmemongkolchai, G. Programmed Death Ligand 1 on Burkholderia pseudomallei –Infected Human Polymorphonuclear Neutrophils Impairs T Cell Functions . *J. Immunol.* (2015). doi:10.4049/jimmunol.1402417
265. Bowers, N. L. *et al.* Immune Suppression by Neutrophils in HIV-1 Infection: Role of PD-L1/PD-1 Pathway. *PLoS Pathog.* (2014). doi:10.1371/journal.ppat.1003993
266. Wang, T. T. *et al.* Tumour-activated neutrophils in gastric cancer foster immune suppression and disease progression through GM-CSF-PD-L1 pathway. *Gut* (2017). doi:10.1136/gutjnl-2016-313075
267. Cheng, Y. *et al.* Cancer-associated fibroblasts induce PDL1+ neutrophils through the IL6-STAT3 pathway that foster immune suppression in hepatocellular carcinoma. *Cell Death Dis.* (2018). doi:10.1038/s41419-018-0458-4
268. Roghanian, A., Drost, E. M., MacNee, W., Howie, S. E. M. & Sallenave, J. M. Inflammatory lung secretions inhibit dendritic cell maturation and function via neutrophil elastase. *Am. J. Respir. Crit. Care Med.* (2006). doi:10.1164/rccm.200605-632OC
269. Odobasic, D. *et al.* Neutrophil myeloperoxidase regulates T-cell2driven tissue inflammation in mice by inhibiting dendritic cell function. *Blood* (2013). doi:10.1182/blood-2012-09-456483
270. Yang, C.-W., Strong, B. S. I., Miller, M. J. & Unanue, E. R. Neutrophils Influence the Level of Antigen Presentation during the Immune Response to Protein Antigens in Adjuvants. *J. Immunol.* (2010). doi:10.4049/jimmunol.1001289
271. Tateosian, N. L. *et al.* Neutrophil elastase treated dendritic cells promote the generation of CD4+FOXP3+ regulatory T cells in vitro. *Cell. Immunol.* (2011). doi:10.1016/j.cellimm.2011.03.013
272. MacManus, C. F. *et al.* VEN-120, a recombinant human lactoferrin, promotes a regulatory T cell [treg] phenotype and drives resolution of inflammation in distinct

murine models of inflammatory bowel disease. *J. Crohn's Colitis* (2017). doi:10.1093/ecco-jcc/jjx056
273. Mishalian, I. *et al.* Neutrophils recruit regulatory T-cells into tumors via secretion of CCL17 - A new mechanism of impaired antitumor immunity. *Int. J. Cancer* (2014). doi:10.1002/ijc.28770
274. Negorev, D. *et al.* Human neutrophils can mimic myeloid-derived suppressor cells (PMN-MDSC) and suppress microbead or lectin-induced T cell proliferation through artefactual mechanisms. *Sci. Rep.* (2018). doi:10.1038/s41598-018-21450-6
275. HULTMARK, D., STEINER, H., RASMUSON, T. & BOMAN, H. G. Insect Immunity. Purification and Properties of Three Inducible Bactericidal Proteins from Hemolymph of Immunized Pupae of Hyalophora cecropia. *Eur. J. Biochem.* (1980). doi:10.1111/j.1432-1033.1980.tb05991.x
276. Zasloff, M. Magainins, a class of antimicrobial peptides from Xenopus skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. U. S. A.* (1987). doi:10.1073/pnas.84.15.5449
277. Kościuczuk, E. M. *et al.* Cathelicidins: family of antimicrobial peptides. A review. *Molecular biology reports* (2012). doi:10.1007/s11033-012-1997-x
278. Gennaro, R., Skerlavaj, B. & Romeo, D. Purification, composition, and activity of two bactenecins, antibacterial peptides of bovine neutrophils. *Infect. Immun.* (1989). doi:10.1128/iai.57.10.3142-3146.1989
279. Cowland, J. B., Johnsen, A. H. & Borregaard, N. hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. *FEBS Lett.* (1995). doi:10.1016/0014-5793(95)00634-L
280. Gallo, R. L. *et al.* Identification of CRAMP, a Cathelin-related Antimicrobial Peptide Expressed in the Embryonic and Adult Mouse* Endogenous antimicrobial peptides play an important role in. *J. Biol. Chem.* (1997).
281. Zanetti, M. Cathelicidins, multifunctional peptides of the innate immunity. *J. Leukoc. Biol.* (2004). doi:10.1189/jlb.0403147
282. Agier, J., Efenberger, M. & Brzezińska-Blaszczyk, E. Cathelicidin impact on inflammatory cells. *Central European Journal of Immunology* (2015). doi:10.5114/ceji.2015.51359
283. Dorschner, R. A. *et al.* Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A streptococcus. *J. Invest. Dermatol.* (2001). doi:10.1046/j.1523-1747.2001.01340.x
284. Frohm, M. *et al.* The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J. Biol. Chem.* (1997). doi:10.1074/jbc.272.24.15258
285. van Harten, R. M., van Woudenbergh, E., van Dijk, A. & Haagsman, H. P. Cathelicidins: Immunomodulatory antimicrobials. *Vaccines* (2018). doi:10.3390/vaccines6030063
286. Sørensen, O., Arnljots, K., Cowland, J. B., Bainton, D. F. & Borregaard, N. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. *Blood* **90**, 2796–2803 (1997).
287. Zanetti, M., Litteri, L., Gennaro, R., Horstmann, H. & Romeo, D. Bactenecins, defense polypeptides of bovine neutrophils, are generated from precursor molecules stored in the large granules. *J. Cell Biol.* (1990). doi:10.1083/jcb.111.4.1363
288. SCOCCHI, M., SKERLAVAJ, B., ROMEO, D. & GENNARO, R. Proteolytic cleavage by

neutrophil elastase converts inactive storage proforms to antibacterial bactenecins. *Eur. J. Biochem.* (1992). doi:10.1111/j.1432-1033.1992.tb17324.x

289. Sørensen, O. E. *et al.* Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* (2001). doi:10.1182/blood.V97.12.3951
290. Sørensen, O. E. *et al.* Processing of seminal plasma hCAP-18 to ALL-38 by gastricsin. A novel mechanism of generating antimicrobial peptides in vagina. *J. Biol. Chem.* (2003). doi:10.1074/jbc.M301608200
291. Yamasaki, K. *et al.* Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. *FASEB J.* (2006). doi:10.1096/fj.06-6075com
292. Yamasaki, K. *et al.* Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea. *Nat. Med.* (2007). doi:10.1038/nm1616
293. Murakami, M., Lopez-Garcia, B., Braff, M., Dorschner, R. A. & Gallo, R. L. Postsecretory Processing Generates Multiple Cathelicidins for Enhanced Topical Antimicrobial Defense. *J. Immunol.* (2004). doi:10.4049/jimmunol.172.5.3070
294. Gombart, A. F. The vitamin D-antimicrobial peptide pathway and its role in protection against infection. *Future Microbiology* (2009). doi:10.2217/fmb.09.87
295. Wang, T.-T. *et al.* Cutting Edge: 1,25-Dihydroxyvitamin D 3 Is a Direct Inducer of Antimicrobial Peptide Gene Expression . *J. Immunol.* (2004). doi:10.4049/jimmunol.173.5.2909
296. Gombart, A. F., Borregaard, N. & Koeffler, H. P. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D 3 . *FASEB J.* (2005). doi:10.1096/fj.04-3284com
297. Schauber, J. *et al.* Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J. Clin. Invest.* (2007). doi:10.1172/JCI30142
298. Aranow, C. Vitamin D and the immune system. in *Journal of Investigative Medicine* (2011). doi:10.2310/JIM.0b013e31821b8755
299. Park, K. *et al.* Regulation of cathelicidin antimicrobial peptide expression by an Endoplasmic Reticulum (ER) stress signaling, vitamin D receptor-independent pathway. *J. Biol. Chem.* (2011). doi:10.1074/jbc.M111.250431
300. Nilsson, M. F. *et al.* The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. *Infect. Immun.* (1999). doi:10.1128/iai.67.5.2561-2566.1999
301. Aberg, K. M. *et al.* Psychological stress downregulates epidermal antimicrobial peptide expression and increases severity of cutaneous infections in mice. *J. Clin. Invest.* (2007). doi:10.1172/JCI31726
302. Seo, S. J. *et al.* The effect of calcipotriol on the expression of human β defensin-2 and LL-37 in cultured human keratinocytes. *Clin. Dev. Immunol.* (2009). doi:10.1155/2009/645898
303. Sperandio, B. *et al.* Virulent Shigella flexneri subverts the host innate immune response through manipulation of antimicrobial peptide gene expression. *J. Exp. Med.* (2008). doi:10.1084/jem.20071698
304. Gennaro, R. & Zanetti, M. Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. *Biopolym. - Pept. Sci. Sect.* (2000). doi:10.1002/1097-0282(2000)55:1<31::AID-BIP40>3.0.CO;2-9
305. Haney, E. F., Straus, S. K. & Hancock, R. E. W. Reassessing the host defense peptide

landscape. *Frontiers in Chemistry* (2019). doi:10.3389/fchem.2019.00043
306. Nizet, V. *et al.* Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* (2001). doi:10.1038/35106587
307. Kovach, M. A. *et al.* Cathelicidin-Related Antimicrobial Peptide Is Required for Effective Lung Mucosal Immunity in Gram-Negative Bacterial Pneumonia. *J. Immunol.* (2012). doi:10.4049/jimmunol.1103196
308. Chromek, M. *et al.* The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection. *Nat. Med.* (2006). doi:10.1038/nm1407
309. Huang, L. C., Reins, R. Y., Gallo, R. L. & McDermott, A. M. Cathelicidin-deficient (Cnlp-/-) mice show increased susceptibility to Pseudomonas aeruginosa keratitis. *Investig. Ophthalmol. Vis. Sci.* (2007). doi:10.1167/iovs.07-0274
310. Ong, P. Y. *et al.* Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* (2002). doi:10.1056/NEJMoa021481
311. Pütsep, K., Carlsson, G., Boman, H. G. & Andersson, M. Deficiency of antibacterial peptides in patients with morbus Kostmann: An observation study. *Lancet* (2002). doi:10.1016/S0140-6736(02)11201-3
312. Wimley, W. C. Describing the mechanism of antimicrobial peptide action with the interfacial activity model. *ACS Chemical Biology* (2010). doi:10.1021/cb1001558
313. Henzler Wildman, K. A., Lee, D. K. & Ramamoorthy, A. Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37. *Biochemistry* (2003). doi:10.1021/bi0273563
314. Xhindoli, D. *et al.* The human cathelicidin LL-37 - A pore-forming antibacterial peptide and host-cell modulator. *Biochim. Biophys. Acta - Biomembr.* (2016). doi:10.1016/j.bbamem.2015.11.003
315. Kirikae, T. *et al.* Protective effects of a human 18-kilodalton cationic antimicrobial protein (CAP18)-derived peptide against murine endotoxemia. *Infect. Immun.* (1998). doi:10.1128/iai.66.5.1861-1868.1998
316. Overhage, J. *et al.* Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect. Immun.* (2008). doi:10.1128/IAI.00318-08
317. Barlow, P. G. *et al.* Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PLoS One* (2011). doi:10.1371/journal.pone.0025333
318. Currie, S. M. *et al.* The Human Cathelicidin LL-37 Has Antiviral Activity against Respiratory Syncytial Virus. *PLoS One* (2013). doi:10.1371/journal.pone.0073659
319. Peter Bergman, Lilian Walter-Jallow, Kristina Broliden, Birgitta Agerberth & Johan Soderlund. The Antimicrobial Peptide LL-37 Inhibits HIV-1 Replication. *Curr. HIV Res.* (2007). doi:10.2174/157016207781023947
320. Tripathi, S. *et al.* The human cathelicidin LL-37 inhibits influenza a viruses through a mechanism distinct from that of surfactant protein d or defensins. *J. Gen. Virol.* (2013). doi:10.1099/vir.0.045013-0
321. Dean, R. E. *et al.* A carpet-based mechanism for direct antimicrobial peptide activity against vaccinia virus membranes. *Peptides* (2010). doi:10.1016/j.peptides.2010.07.028
322. López-García, B., Lee, P. H. A., Yamasaki, K. & Gallo, R. L. Anti-fungal activity of cathelicidins and their potential role in Candida albicans skin infection. *J. Invest. Dermatol.* (2005). doi:10.1111/j.0022-202X.2005.23713.x
323. Den Hertog, A. L. *et al.* Candidacidal effects of two antimicrobial peptides: Histatin 5 causes small membrane defects, but LL-37 causes massive disruption of the cell

membrane. *Biochem. J.* (2005). doi:10.1042/BJ20042099
324. Wong, J. H. *et al.* Antifungal action of human cathelicidin fragment (LL13-37) on Candida albicans. *Peptides* (2011). doi:10.1016/j.peptides.2011.08.018
325. Luo, X. L. *et al.* LL37 inhibits aspergillus fumigatus infection via directly binding to the fungus and preventing excessive inflammation. *Front. Immunol.* (2019). doi:10.3389/fimmu.2019.00283
326. Vandamme, D., Landuyt, B., Luyten, W. & Schoofs, L. A comprehensive summary of LL-37, the factoctum human cathelicidin peptide. *Cellular Immunology* (2012). doi:10.1016/j.cellimm.2012.11.009
327. De Yang, B. *et al.* LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J. Exp. Med.* (2000). doi:10.1084/jem.192.7.1069
328. Zhang, Z. *et al.* Evidence that cathelicidin peptide LL-37 may act as a functional ligand for CXCR2 on human neutrophils. *Eur. J. Immunol.* (2009). doi:10.1002/eji.200939496
329. Ferrari, D. *et al.* The P2X 7 receptor: a key player in IL-1 processing and release . *J. Immunol.* (2007). doi:10.4049/jimmunol.179.12.8569-b
330. Tomasinsig, L. *et al.* The human cathelicidin LL-37 modulates the activities of the P2X 7 receptor in a structure-dependent manner. *J. Biol. Chem.* (2008). doi:10.1074/jbc.M802185200
331. Tjabringa, G. S. *et al.* The Antimicrobial Peptide LL-37 Activates Innate Immunity at the Airway Epithelial Surface by Transactivation of the Epidermal Growth Factor Receptor. *J. Immunol.* (2003). doi:10.4049/jimmunol.171.12.6690
332. Tokumaru, S. *et al.* Induction of Keratinocyte Migration via Transactivation of the Epidermal Growth Factor Receptor by the Antimicrobial Peptide LL-37. *J. Immunol.* (2005). doi:10.4049/jimmunol.175.7.4662
333. Sandgren, S. *et al.* The Human Antimicrobial Peptide LL-37 Transfers Extracellular DNA Plasmid to the Nuclear Compartment of Mammalian Cells via Lipid Rafts and Proteoglycan-dependent Endocytosis. *J. Biol. Chem.* (2004). doi:10.1074/jbc.M311440200
334. Lande, R. *et al.* Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* (2007). doi:10.1038/nature06116
335. van der Does, A. M. *et al.* LL-37 Directs Macrophage Differentiation toward Macrophages with a Proinflammatory Signature. *J. Immunol.* (2010). doi:10.4049/jimmunol.1000376
336. Davidson, D. J. *et al.* The Cationic Antimicrobial Peptide LL-37 Modulates Dendritic Cell Differentiation and Dendritic Cell-Induced T Cell Polarization. *J. Immunol.* **172**, 1146–1156 (2004).
337. Hurtado, P. & Au Peh, C. LL-37 Promotes Rapid Sensing of CpG Oligodeoxynucleotides by B Lymphocytes and Plasmacytoid Dendritic Cells. *J. Immunol.* (2010). doi:10.4049/jimmunol.0902305
338. Findlay, E. G. *et al.* Exposure to the antimicrobial peptide LL-37 produces dendritic cells optimized for immunotherapy. *Oncoimmunology* (2019). doi:10.1080/2162402X.2019.1608106
339. Lande, R. *et al.* The antimicrobial peptide LL37 is a T-cell autoantigen in psoriasis. *Nat. Commun.* (2014). doi:10.1038/ncomms6621
340. Thomi, R. *et al.* Elevated levels of the antimicrobial peptide LL-37 in hidradenitis

suppurativa are associated with a Th1/Th17 immune response. *Exp. Dermatol.* (2018). doi:10.1111/exd.13482

341. Kurosaka, K., Chen, Q., Yarovinsky, F., Oppenheim, J. J. & Yang, D. Mouse Cathelin-Related Antimicrobial Peptide Chemoattracts Leukocytes Using Formyl Peptide Receptor-Like 1/Mouse Formyl Peptide Receptor-Like 2 as the Receptor and Acts as an Immune Adjuvant. *J. Immunol.* (2005). doi:10.4049/jimmunol.174.10.6257
342. Lau, Y. E. *et al.* Interaction and cellular localization of the human host defense peptide LL-37 with lung epithelial cells. *Infect. Immun.* (2005). doi:10.1128/IAI.73.1.583-591.2005
343. Beaumont, P. E. *et al.* Cathelicidin host defence peptide augments clearance of pulmonary Pseudomonas aeruginosa infection by its influence on neutrophil function in vivo. *PLoS One* (2014). doi:10.1371/journal.pone.0099029
344. Choi, K. Y. G., Napper, S. & Mookherjee, N. Human cathelicidin LL-37 and its derivative IG-19 regulate interleukin-32-induced inflammation. *Immunology* (2014). doi:10.1111/imm.12291
345. Larrick, J. W. *et al.* Human CAP18: A novel antimicrobial lipopolysaccharide-binding protein. *Infect. Immun.* (1995). doi:10.1128/iai.63.4.1291-1297.1995
346. Mookherjee, N. *et al.* Modulation of the TLR-Mediated Inflammatory Response by the Endogenous Human Host Defense Peptide LL-37. *J. Immunol.* (2006). doi:10.4049/jimmunol.176.4.2455
347. Kandler, K. *et al.* The anti-microbial peptide LL-37 inhibits the activation of dendritic cells by TLR ligands. *Int. Immunol.* (2006). doi:10.1093/intimm/dxl107
348. Di Nardo, A. *et al.* Cathelicidin Antimicrobial Peptides Block Dendritic Cell TLR4 Activation and Allergic Contact Sensitization. *J. Immunol.* (2007). doi:10.4049/jimmunol.178.3.1829
349. Tjabringa, G. S., Ninaber, D. K., Drijfhout, J. W., Rabe, K. F. & Hiemstra, P. S. Human cathelicidin LL-37 Is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *Int. Arch. Allergy Immunol.* (2006). doi:10.1159/000092305
350. De Yang *et al.* Ll-37, the Neutrophil Granule–And Epithelial Cell–Derived Cathelicidin, Utilizes Formyl Peptide Receptor–Like 1 (Fprl1) as a Receptor to Chemoattract Human Peripheral Blood Neutrophils, Monocytes, and T Cells. *J. Exp. Med.* (2000). doi:10.1084/jem.192.7.1069
351. Yang, D., Chen, Q., Le, Y., Wang, J. M. & Oppenheim, J. J. Differential Regulation of Formyl Peptide Receptor-Like 1 Expression During the Differentiation of Monocytes to Dendritic Cells and Macrophages. *J. Immunol.* (2001). doi:10.4049/jimmunol.166.6.4092
352. Agerberth, B. *et al.* The human antimicrobial and chemotactic peptides LL-37 and α-defensins are expressed by specific lymphocyte and monocyte populations. *Blood* (2000). doi:10.1182/blood.v96.9.3086.h8003086_3086_3093
353. Scott, M. G., Davidson, D. J., Gold, M. R., Bowdish, D. & Hancock, R. E. W. The Human Antimicrobial Peptide LL-37 Is a Multifunctional Modulator of Innate Immune Responses. *J. Immunol.* (2002). doi:10.4049/jimmunol.169.7.3883
354. Coorens, M., Scheenstra, M. R., Veldhuizen, E. J. A. & Haagsman, H. P. Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis. *Sci. Rep.* (2017). doi:10.1038/srep40874
355. Nagaoka, I., Tamura, H. & Hirata, M. An Antimicrobial Cathelicidin Peptide, Human

CAP18/LL-37, Suppresses Neutrophil Apoptosis via the Activation of Formyl-Peptide Receptor-Like 1 and P2X 7 . *J. Immunol.* (2006). doi:10.4049/jimmunol.176.5.3044
356. Otte, J. M. *et al.* Effects of the cathelicidin LL-37 on intestinal epithelial barrier integrity. *Regul. Pept.* (2009). doi:10.1016/j.regpep.2009.03.009
357. Kim, H. J. *et al.* LL-37 suppresses sodium nitroprusside-induced apoptosis of systemic sclerosis dermal fibroblasts. *Experimental Dermatology* (2011). doi:10.1111/j.1600-0625.2011.01327.x
358. Chamorro, C. I., Weber, G., Grönberg, A., Pivarcsi, A. & Ståhle, M. The human antimicrobial peptide LL-37 suppresses apoptosis in keratinocytes. *J. Invest. Dermatol.* (2009). doi:10.1038/jid.2008.321
359. Mader, J. S., Marcet-Palacios, M., Hancock, R. E. W. & Bleackley, R. C. The human cathelicidin, LL-37, induces granzyme-mediated apoptosis in cytotoxic T lymphocytes. *Exp. Cell Res.* (2011). doi:10.1016/j.yexcr.2010.11.015
360. Mader, J. S., Ewen, C., Hancock, R. E. W. & Bleackley, R. C. The human cathelicidin, LL-37, induces granzyme-mediated apoptosis in regulatory T cells. *J. Immunother.* (2011). doi:10.1097/CJI.0b013e318207ecdf
361. Yasuda, K., Takeuchi, Y. & Hirota, K. The pathogenicity of Th17 cells in autoimmune diseases. *Seminars in Immunopathology* (2019). doi:10.1007/s00281-019-00733-8
362. Langrish, C. L. *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* (2005). doi:10.1084/jem.20041257
363. Gaffen, S. L., Jain, R., Garg, A. V. & Cua, D. J. IL-23-IL-17 immune axis: Discovery, Mechanistic Understanding, and Clinical Testing. *Nat. Rev. Immunol.* (2014). doi:10.1038/NRI3707
364. Kahlenberg, J. M. & Kaplan, M. J. Little Peptide, Big Effects: The Role of LL-37 in Inflammation and Autoimmune Disease. *J. Immunol.* **191**, 4895–4901 (2013).
365. Paulsen, F. *et al.* Antimicrobial peptides are expressed and produced in healthy and inflamed human synovial membranes. *J. Pathol.* (2002). doi:10.1002/path.1224
366. Säll, J. *et al.* The antimicrobial peptide LL-37 alters human osteoblast Ca handling and induces Ca2+-independent apoptosis. *J. Innate Immun.* (2013). doi:10.1159/000346587
367. Morizane, S. & Gallo, R. L. Antimicrobial peptides in the pathogenesis of psoriasis. *J. Dermatol.* (2012). doi:10.1111/j.1346-8138.2011.01483.x
368. Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* (2006). doi:10.1038/nature04753
369. Warnock, R. A., Askari, S., Butcher, E. C. & Von Andrian, U. H. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. *J. Exp. Med.* (1998). doi:10.1084/jem.187.2.205
370. Schumann, J., Stanko, K., Schliesser, U., Appelt, C. & Sawitzki, B. Differences in CD44 surface expression levels and function discriminates IL-17 and IFN-γ producing helper T cells. *PLoS One* (2015). doi:10.1371/journal.pone.0132479
371. Kreymborg, K. *et al.* IL-22 Is Expressed by Th17 Cells in an IL-23-Dependent Fashion, but Not Required for the Development of Autoimmune Encephalomyelitis. *J. Immunol.* (2007). doi:10.4049/jimmunol.179.12.8098
372. Hensel, J. A., Khattar, V., Ashton, R. & Ponnazhagan, S. Characterization of immune cell subtypes in three commonly used mouse strains reveals gender and strain-specific variations. *Lab. Investig.* (2019). doi:10.1038/s41374-018-0137-1
373. Cobb, D. & Smeltz, R. B. Regulation of Proinflammatory Th17 Responses during

Trypanosoma cruzi Infection by IL-12 Family Cytokines . *J. Immunol.* (2012). doi:10.4049/jimmunol.1103478

374. Prochazkova, J., Pokorna, K. & Holan, V. IL-12 inhibits the TGF-β-dependent T cell developmental programs and skews the TGF-β-induced differentiation into a Th1-like direction. *Immunobiology* (2012). doi:10.1016/j.imbio.2011.07.032
375. Davidson, D. J. *et al.* The Cationic Antimicrobial Peptide LL-37 Modulates Dendritic Cell Differentiation and Dendritic Cell-Induced T Cell Polarization. *J. Immunol.* (2004). doi:10.4049/jimmunol.172.2.1146
376. Tullett M., K. M., Lahoud H., M. H. & Radford J., K. J. Harnessing human cross-presenting CLEC9A+XCR1+ dendritic cells for immunotherapy. *Front. Immunol.* (2014). doi:10.3389/fimmu.2014.00239
377. Wenzel, U. A., Jonstrand, C., Hansson, G. C. & Wick, M. J. CD103+CD11b+ dendritic cells induce Th17 T cells in Muc2-deficient mice with extensively spread colitis. *PLoS One* (2015). doi:10.1371/journal.pone.0130750
378. Mader, J. S., Marcet-Palacios, M., Hancock, R. E. W. & Bleackley, R. C. The human cathelicidin, LL-37, induces granzyme-mediated apoptosis in cytotoxic T lymphocytes. *Exp. Cell Res.* **317**, 531–538 (2011).
379. Vermes, I., Haanen, C., Steffens-Nakken, H. & Reutellingsperger, C. A novel assay for apoptosis Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* (1995). doi:10.1016/0022-1759(95)00072-I
380. Boulos, L., Prévost, M., Barbeau, B., Coallier, J. & Desjardins, R. LIVE/DEAD(®) BacLight(TM): Application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J. Microbiol. Methods* (1999). doi:10.1016/S0167-7012(99)00048-2
381. Annunziato, F. *et al.* Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* (2007). doi:10.1084/jem.20070663
382. Acosta-Rodriguez, E. V. *et al.* Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* (2007). doi:10.1038/ni1467
383. Dunay, G. A. *et al.* Parallel assessment of Th17 cell frequencies by surface marker co-expression versus ex vivo IL-17 production in HIV-1 infection. *Cytom. Part B - Clin. Cytom.* (2016). doi:10.1002/cyto.b.21352
384. Lyons, A. B. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J. Immunol. Methods* (2000). doi:10.1016/S0022-1759(00)00231-3
385. Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. & Stockinger, B. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* (2006). doi:10.1016/j.immuni.2006.01.001
386. Hirahara, K. *et al.* Signal transduction pathways and transcriptional regulation in Th17 cell differentiation. *Cytokine Growth Factor Rev.* (2010). doi:10.1016/j.cytogfr.2010.10.006
387. Vandamme, D., Landuyt, B., Luyten, W. & Schoofs, L. A comprehensive summary of LL-37, the factoctum human cathelicidin peptide. *Cell. Immunol.* **280**, 22–35 (2012).
388. Pestonjamasp, V. K., Huttner, K. H. & Gallo, R. L. Processing site and gene structure for the murine antimicrobial peptide CRAMP. *Peptides* (2001). doi:10.1016/S0196-9781(01)00499-5
389. Stockinger, B. & Veldhoen, M. Differentiation and function of Th17 T cells. *Current*

Opinion in Immunology (2007). doi:10.1016/j.coi.2007.04.005
390. Bedoya, S. K., Wilson, T. D., Collins, E. L., Lau, K. & Larkin, J. Isolation and th17 differentiation of naïve CD4 T lymphocytes. *J. Vis. Exp.* (2013). doi:10.3791/50765
391. Schaller-Bals, S., Schulze, A. & Bals, R. Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. *Am. J. Respir. Crit. Care Med.* (2002). doi:10.1164/ajrccm.165.7.200110-020
392. Simon, S. & Labarriere, N. PD-1 expression on tumor-specific T cells: Friend or foe for immunotherapy? *Oncoimmunology* (2017). doi:10.1080/2162402X.2017.1364828
393. Goods, B. A. *et al.* Functional differences between PD-1+ and PD-1- CD4+ effector T cells in healthy donors and patients with glioblastoma multiforme. *PLoS One* (2017). doi:10.1371/journal.pone.0181538
394. Chen, S. *et al.* Mechanisms regulating PD-L1 expression on tumor and immune cells. *J. Immunother. Cancer* (2019). doi:10.1186/s40425-019-0770-2
395. Xu-Monette, Z. Y., Zhang, M., Li, J. & Young, K. H. PD-1/PD-L1 blockade: Have we found the key to unleash the antitumor immune response? *Frontiers in Immunology* (2017). doi:10.3389/fimmu.2017.01597
396. Kawaguchi, M., Adachi, M., Oda, N., Kokubu, F. & Huang, S. K. IL-17 cytokine family. *Journal of Allergy and Clinical Immunology* (2004). doi:10.1016/j.jaci.2004.10.019
397. Chang, S. H. & Dong, C. IL-17F: Regulation, signaling and function in inflammation. *Cytokine* (2009). doi:10.1016/j.cyto.2008.12.024
398. Wanke, F. *et al.* Expression of IL-17F is associated with non-pathogenic Th17 cells. *J. Mol. Med.* (2018). doi:10.1007/s00109-018-1662-5
399. Piccinni, M. P. *et al.* Medroxyprogesterone acetate decreases Th1, Th17, and increases Th22 responses via AhR signaling which could affect susceptibility to infections and inflammatory disease. *Front. Immunol.* (2019). doi:10.3389/fimmu.2019.00642
400. Plé, C. *et al.* Polycyclic aromatic hydrocarbons reciprocally regulate IL-22 and IL-17 cytokines in peripheral blood mononuclear cells from both healthy and asthmatic subjects. *PLoS One* (2015). doi:10.1371/journal.pone.0122372
401. Sutton, C., Brereton, C., Keogh, B., Mills, K. H. G. & Lavelle, E. C. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J. Exp. Med.* (2006). doi:10.1084/jem.20060285
402. Lee, Y. *et al.* Induction and molecular signature of pathogenic T H 17 cells. *Nat. Immunol.* (2012). doi:10.1038/ni.2416
403. Esplugues, E. *et al.* Control of TH17 cells occurs in the small intestine. *Nature* (2011). doi:10.1038/nature10228
404. Bettelli, E., Oukka, M. & Kuchroo, V. K. TH-17 cells in the circle of immunity and autoimmunity. *Nat. Immunol.* (2007). doi:10.1038/ni0407-345
405. Balasubramani, A., Mukasa, R., Hatton, R. D. & Weaver, C. T. Regulation of the Ifng locus in the context of T-lineage specification and plasticity. *Immunol. Rev.* (2010). doi:10.1111/j.1600-065X.2010.00961.x
406. Eri, R., McGuckin, M. A. & Wadley, R. T cell transfer model of colitis: A great tool to assess the contribution of T cells in chronic intestinal inflammation. *Methods Mol. Biol.* (2012). doi:10.1007/978-1-61779-527-5_19
407. Zelante, T. *et al.* $CD103^+$ Dendritic Cells Control Th17 Cell Function in the Lung. *Cell Rep.* (2015). doi:10.1016/j.celrep.2015.08.030
408. Khayrullina, T., Yen, J.-H., Jing, H. & Ganea, D. In Vitro Differentiation of Dendritic Cells in the Presence of Prostaglandin E 2 Alters the IL-12/IL-23 Balance and

Promotes Differentiation of Th17 Cells . *J. Immunol.* (2008). doi:10.4049/jimmunol.181.1.721
409. Wang, Y. & Rothstein, T. L. Induction of Th17 cell differentiation by B-1 cells. *Front. Immunol.* (2012). doi:10.3389/fimmu.2012.00281
410. Barr, T. A. *et al.* B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J. Exp. Med.* (2012). doi:10.1084/jem.20111675
411. Jones, G. W. *et al.* Loss of CD4 + T Cell IL-6R Expression during Inflammation Underlines a Role for IL-6 Trans Signaling in the Local Maintenance of Th17 Cells . *J. Immunol.* (2010). doi:10.4049/jimmunol.0901528
412. Zhang, F. *et al.* Elevated IL-6 receptor expression on CD4+ T cells contributes to the increased Th17 responses in patients with chronic hepatitis B. *Virol. J.* (2011). doi:10.1186/1743-422X-8-270
413. Fuseini, H. *et al.* ERα Signaling Increased IL-17A Production in Th17 Cells by Upregulating IL-23R Expression, Mitochondrial Respiration, and Proliferation. *Front. Immunol.* (2019). doi:10.3389/fimmu.2019.02740
414. Bei, Y. *et al.* Cathelicidin-related antimicrobial peptide protects against myocardial ischemia/reperfusion injury. *BMC Med.* (2019). doi:10.1186/s12916-019-1268-y
415. Suzuki, K. *et al.* Human anti-microbial cathelicidin peptide LL-37 suppresses the LPS-induced apoptosis of endothelial cells. *Int. Immunol.* (2011). doi:10.1093/intimm/dxq471
416. Nagaoka, I., Suzuki, K., Niyonsaba, F., Tamura, H. & Hirata, M. Modulation of Neutrophil Apoptosis by Antimicrobial Peptides. *ISRN Microbiol.* (2012). doi:10.5402/2012/345791
417. Björstad, Å. *et al.* The host defense peptide LL-37 selectively permeabilizes apoptotic leukocytes. *Antimicrob. Agents Chemother.* (2009). doi:10.1128/AAC.01310-08
418. Wei, L., Laurence, A., Elias, K. M. & O'Shea, J. J. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J. Biol. Chem.* (2007). doi:10.1074/jbc.M705100200
419. Kleinewietfeld, M. & Hafler, D. A. The plasticity of human Treg and Th17 cells and its role in autoimmunity. *Seminars in Immunology* (2013). doi:10.1016/j.smim.2013.10.009
420. Gagliani, N. *et al.* TH17 cells transdifferentiate into regulatory T cells uring resolution of inflammation. *Nature* (2015). doi:10.1038/nature14452
421. Lee, Y. K. *et al.* Late Developmental Plasticity in the T Helper 17 Lineage. *Immunity* (2009). doi:10.1016/j.immuni.2008.11.005
422. Bauché, D. & Marie, J. C. Transforming growth factor β: A master regulator of the gut microbiota and immune cell interactions. *Clinical and Translational Immunology* (2017). doi:10.1038/cti.2017.9
423. Zheng, X. *et al.* Cathelicidin-related antimicrobial peptide protects against cardiac fibrosis in diabetic mice heart by regulating endothelial-mesenchymal transition. *Int. J. Biol. Sci.* (2019). doi:10.7150/ijbs.35736
424. Duplantier, A. J. & van Hoek, M. L. The human cathelicidin antimicrobial peptide LL-37 as a potential treatment for polymicrobial infected wounds. *Frontiers in Immunology* (2013). doi:10.3389/fimmu.2013.00143
425. Gambade, A. *et al.* Activation of TRPV2 and BKCa channels by the LL-37 enantiomers stimulates calcium entry and migration of cancer cells. *Oncotarget* **7**, 23785–800 (2016).
426. Gordon, Y. J. *et al.* Human cathelicidin (LL-37), a multifunctional peptide, is

expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. *Curr. Eye Res.* (2005). doi:10.1080/02713680590934111
427. Muñoz, M. *et al.* Antimicrobial peptide LL-37 participates in the transcriptional regulation of melanoma cells. *J. Cancer* (2016). doi:10.7150/JCA.16947
428. Sun, L., Fu, J. & Zhou, Y. Metabolism controls the balance of Th17/T-regulatory cells. *Frontiers in Immunology* (2017). doi:10.3389/fimmu.2017.01632
429. Shi, L. Z. *et al.* HIF1α-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J. Exp. Med.* (2011). doi:10.1084/jem.20110278
430. Dang, E. V. *et al.* Control of TH17/Treg balance by hypoxia-inducible factor 1. *Cell* (2011). doi:10.1016/j.cell.2011.07.033
431. Delgoffe, G. M. *et al.* The mammalian Target of Rapamycin (mTOR) regulates T helper cell differentiation through the selective activation of mTORC1 and mTORC2 signaling. *Nat. Immunol.* (2011). doi:10.1038/ni.2005.The
432. Sun, W. *et al.* LL-37 attenuates inflammatory impairment via mTOR signaling-dependent mitochondrial protection. *Int. J. Biochem. Cell Biol.* (2014). doi:10.1016/j.biocel.2014.06.015
433. Mookherjee, N. *et al.* Intracellular Receptor for Human Host Defense Peptide LL-37 in Monocytes. *J. Immunol.* (2009). doi:10.4049/jimmunol.0802586
434. Zhang, S. The role of transforming growth factor β in T helper 17 differentiation. *Immunology* (2018). doi:10.1111/imm.12938
435. Zhang, S. *et al.* Reversing SKI-SMAD4-mediated suppression is essential for TH17 cell differentiation. *Nature* (2017). doi:10.1038/nature24283
436. Biswas, P. S. *et al.* Phosphorylation of IRF4 by ROCK2 regulates IL-17 and IL-21 production and the development of autoimmunity in mice. *J. Clin. Invest.* (2010). doi:10.1172/JCI42856
437. Ichiyama, K. *et al.* Transcription Factor Smad-Independent T Helper 17 Cell Induction by Transforming-Growth Factor-β Is Mediated by Suppression of Eomesodermin. *Immunity* (2011). doi:10.1016/j.immuni.2011.02.021
438. Carow, B. & Rottenberg, M. E. SOCS3, a major regulator of infection and inflammation. *Frontiers in Immunology* (2014). doi:10.3389/fimmu.2014.00058
439. Yu, Q., Sharma, A., Ghosh, A. & Sen, J. M. T Cell Factor-1 Negatively Regulates Expression of IL-17 Family of Cytokines and Protects Mice from Experimental Autoimmune Encephalomyelitis. *J. Immunol.* (2011). doi:10.4049/jimmunol.1003497
440. Ouyang, X. *et al.* Transcription factor IRF8 directs a silencing programme for TH17 cell differentiation. *Nat. Commun.* **2**, 312–314 (2011).
441. Stockinger, B. Good for Goose, but Not for Gander: IL-2 Interferes with Th17 Differentiation. *Immunity* (2007). doi:10.1016/j.immuni.2007.03.001
442. Laurence, A. *et al.* Interleukin-2 Signaling via STAT5 Constrains T Helper 17 Cell Generation. *Immunity* (2007). doi:10.1016/j.immuni.2007.02.009
443. Kallies, A. & Good-Jacobson, K. L. Transcription Factor T-bet Orchestrates Lineage Development and Function in the Immune System. *Trends in Immunology* (2017). doi:10.1016/j.it.2017.02.003
444. Ghoreschi, K. *et al.* Generation of pathogenic TH 17 cells in the absence of TGF-β 2 signalling. *Nature* (2010). doi:10.1038/nature09447
445. Rothhammer, V. & Quintana, F. J. The aryl hydrocarbon receptor: an environmental sensor integrating immune responses in health and disease. *Nature Reviews Immunology* (2019). doi:10.1038/s41577-019-0125-8

446. Baricza, E., Tamási, V., Marton, N., Buzás, E. I. & Nagy, G. The emerging role of aryl hydrocarbon receptor in the activation and differentiation of Th17 cells. *Cell. Mol. Life Sci.* **73**, 95–117 (2016).
447. Zhao, B., DeGroot, D. E., Hayashi, A., He, G. & Denison, M. S. Ch223191 is a ligand-selective antagonist of the Ah (dioxin) receptor. *Toxicol. Sci.* (2010). doi:10.1093/toxsci/kfq217
448. Quintana, F. J. *et al.* Control of Treg and TH17 cell differentiation by the aryl hydrocarbon receptor. *Nature* (2008). doi:10.1038/nature06880
449. Hoover, D. M. *et al.* The structure of human β-defensin-2 shows evidence of higher order oligomerization. *J. Biol. Chem.* (2000). doi:10.1074/jbc.M006098200
450. Bai, X. & Chen, X. Rational design, conformational analysis and membrane-penetrating dynamics study of Bac2A-derived antimicrobial peptides against gram-positive clinical strains isolated from pyemia. *J. Theor. Biol.* (2019). doi:10.1016/j.jtbi.2019.03.018
451. Zhang, J. *et al.* IκB Kinase ε Is an NFATc1 Kinase that Inhibits T Cell Immune Response. *Cell Rep.* (2016). doi:10.1016/j.celrep.2016.05.083
452. Tai, X. G. *et al.* A role for CD9 molecules in T cell activation. *J. Exp. Med.* (1996). doi:10.1084/jem.184.2.753
453. Tai, X. G. *et al.* CD9-Mediated Costimulation of TCR-Triggered Naive T Cells Leads to Activation Followed by Apoptosis. *J. Immunol.* (1997).
454. Lenardo, M. J. Interleukin-2 programs mouse αβ T lymphocytes for apoptosis. *Nature* (1991). doi:10.1038/353858a0
455. Qiu, J. *et al.* The Aryl Hydrocarbon Receptor Regulates Gut Immunity through Modulation of Innate Lymphoid Cells. *Immunity* (2012). doi:10.1016/j.immuni.2011.11.011
456. Kischkel, F. C. *et al.* Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* (2000). doi:10.1016/S1074-7613(00)80212-5
457. Zhang, S., Lukacs, N. W., Lawless, V. A., Kunkel, S. L. & Kaplan, M. H. Cutting Edge: Differential Expression of Chemokines in Th1 and Th2 Cells Is Dependent on Stat6 But Not Stat4. *J. Immunol.* (2000). doi:10.4049/jimmunol.165.1.10
458. Tang, W. *et al.* The Oncoprotein and Transcriptional Regulator Bcl-3 Governs Plasticity and Pathogenicity of Autoimmune T Cells. *Immunity* (2014). doi:10.1016/j.immuni.2014.09.017
459. Giese, N. A. *et al.* Interferon (IFN) consensus sequence-binding protein, a transcription factor of the IFN regulatory factor family, regulates immune responses in vivo through control of interleukin 12 expression. *J. Exp. Med.* (1997). doi:10.1084/jem.186.9.1535
460. Read, K. A. *et al.* Integrated STAT3 and Ikaros Zinc Finger Transcription Factor Activities Regulate Bcl-6 Expression in CD4 + Th Cells . *J. Immunol.* (2017). doi:10.4049/jimmunol.1700106
461. Rieder, S. A. *et al.* Eos Is Redundant for Regulatory T Cell Function but Plays an Important Role in IL-2 and Th17 Production by CD4 $^{+}$ Conventional T Cells. *J. Immunol.* (2015). doi:10.4049/jimmunol.1500627
462. Brabletz, T. *et al.* Transforming growth factor beta and cyclosporin A inhibit the inducible activity of the interleukin-2 gene in T cells through a noncanonical octamer-binding site. *Mol. Cell. Biol.* (1993). doi:10.1128/mcb.13.2.1155
463. Goodman, W. A., Young, A. B., McCormick, T. S., Cooper, K. D. & Levine, A. D. Stat3

Phosphorylation Mediates Resistance of Primary Human T Cells to Regulatory T Cell Suppression. *J. Immunol.* (2011). doi:10.4049/jimmunol.1001455

464. Tanaka, K. *et al.* Loss of Suppressor of Cytokine Signaling 1 in Helper T Cells Leads to Defective Th17 Differentiation by Enhancing Antagonistic Effects of IFN-γ on STAT3 and Smads. *J. Immunol.* (2008). doi:10.4049/jimmunol.180.6.3746
465. Szabo, S. J. *et al.* A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* (2000). doi:10.1016/S0092-8674(00)80702-3
466. Lazarevic, V. *et al.* T-bet represses TH 17 differentiation by preventing Runx1-mediated activation of the gene encoding RORγt. *Nat. Immunol.* (2011). doi:10.1038/ni.1969
467. Egawa, T. *et al.* Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. *Nat. Immunol.* (2007). doi:10.1084/jem.20070133
468. Yeh, W. I., McWilliams, I. L. & Harrington, L. E. IFNγ inhibits Th17 differentiation and function via Tbet-dependent and Tbet-independent mechanisms. *J. Neuroimmunol.* (2014). doi:10.1016/j.jneuroim.2013.12.001
469. Park, I.-K., Shultz, L. D., Letterio, J. J. & Gorham, J. D. TGF-β1 Inhibits T-bet Induction by IFN-γ in Murine CD4 + T Cells through the Protein Tyrosine Phosphatase Src Homology Region 2 Domain-Containing Phosphatase-1 . *J. Immunol.* (2005). doi:10.4049/jimmunol.175.9.5666
470. Bhaumik, S. & Basu, R. Cellular and molecular dynamics of Th17 differentiation and its developmental plasticity in the intestinal immune response. *Frontiers in Immunology* (2017). doi:10.3389/fimmu.2017.00254
471. Kebir, H. *et al.* Preferential recruitment of interferon-γ-expressing TH17 cells in multiple sclerosis. *Ann. Neurol.* (2009). doi:10.1002/ana.21748
472. Grifka-Walk, H. M. & Segal, B. M. T-bet promotes the accumulation of encephalitogenic Th17 cells in the CNS. *J. Neuroimmunol.* (2017). doi:10.1016/j.jneuroim.2016.05.007
473. Liu, X. *et al.* The role of STAT3 and AhR in the differentiation of CD4 + T cells into Th17 and Treg cells. *Med. (United States)* (2017). doi:10.1097/MD.0000000000006615
474. Duarte, J. H., Di Meglio, P., Hirota, K., Ahlfors, H. & Stockinger, B. Differential influences of the aryl hydrocarbon receptor on Th17 mediated responses in vitro and in vivo. *PLoS One* (2013). doi:10.1371/journal.pone.0079819
475. de Lima, K. A. *et al.* TGFβ1 signaling sustains aryl hydrocarbon receptor (AHR) expression and restrains the pathogenic potential of TH17 cells by an AHR-independent mechanism. *Cell Death Dis.* (2018). doi:10.1038/s41419-018-1107-7
476. Harrington, L. E. *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* (2005). doi:10.1038/ni1254
477. Ohtake, F. *et al.* Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. *Nature* (2007). doi:10.1038/nature05683
478. Quintana, F. J. *et al.* Aiolos promotes T H17 differentiation by directly silencing Il2 expression. *Nat. Immunol.* (2012). doi:10.1038/ni.2363
479. Wu, X., Tian, J. & Wang, S. Insight into non-pathogenic Th17 cells in autoimmune diseases. *Frontiers in Immunology* (2018). doi:10.3389/fimmu.2018.01112
480. Zhang, L., Yuan, S., Cheng, G. & Guo, B. Type I IFN promotes IL-10 production from T cells to suppress Th17 cells and Th17-associated autoimmune inflammation. *PLoS One* (2011). doi:10.1371/journal.pone.0028432

481. Gaublomme, J. T. *et al.* Single-Cell Genomics Unveils Critical Regulators of Th17 Cell Pathogenicity. *Cell* (2015). doi:10.1016/j.cell.2015.11.009
482. Zheng, W. *et al.* Up-Regulation of Hlx in Immature Th Cells Induces IFN-γ Expression. *J. Immunol.* (2004). doi:10.4049/jimmunol.172.1.114
483. Yang, Y. *et al.* T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *J. Exp. Med.* (2009). doi:10.1084/jem.20082584
484. Apetoh, L. *et al.* The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat. Immunol.* (2010). doi:10.1038/ni.1912
485. Findlay, B., Zhanel, G. G. & Schweizer, F. Cationic amphiphiles, a new generation of antimicrobials inspired by the natural antimicrobial peptide scaffold. *Antimicrobial Agents and Chemotherapy* (2010). doi:10.1128/AAC.00530-10
486. Jones, M. K., Anantharamaiah, G. M. & Segrest, J. P. Computer programs to identify and classify amphipathic α helical domains. *J. Lipid Res.* (1992).
487. Sawai, M. V *et al.* The NMR structure of human beta-defensin-2 reveals a novel alpha-helical segment. *Biochemistry* (2001).
488. Friedrich, C. L., Rozek, A., Patrzykat, A. & Hancock, R. E. W. Structure and Mechanism of Action of an Indolicidin Peptide Derivative with Improved Activity against Gram-positive Bacteria. *J. Biol. Chem.* (2001). doi:10.1074/jbc.M009691200
489. Kin, N. W., Chen, Y., Stefanov, E. K., Gallo, R. L. & Kearney, J. F. Cathelin-related antimicrobial peptide differentially regulates T- and B-cell function. *Eur. J. Immunol.* (2011). doi:10.1002/eji.201141606
490. Pelletier, M. *et al.* Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood* (2010). doi:10.1182/blood-2009-04-216085
491. Kolls, J. K. & Lindén, A. Interleukin-17 family members and inflammation. *Immunity* (2004). doi:10.1016/j.immuni.2004.08.018
492. Thewissen, M., Damoiseaux, J., van de Gaar, J. & Tervaert, J. W. C. Neutrophils and T cells: Bidirectional effects and functional interferences. *Mol. Immunol.* **48**, 2094–2101 (2011).
493. Trentini, M. M., de Oliveira, F. M., Kipnis, A. & Junqueira-Kipnis, A. P. The role of neutrophils in the induction of specific Th1 and Th17 during Vaccination against tuberculosis. *Front. Microbiol.* (2016). doi:10.3389/fmicb.2016.00898
494. Lambert, S. *et al.* Neutrophil Extracellular Traps Induce Human Th17 Cells: Effect of Psoriasis-Associated TRAF3IP2 Genotype. *J. Invest. Dermatol.* (2019). doi:10.1016/j.jid.2018.11.021
495. Chen, W., Wang, Q., Ke, Y. & Lin, J. Neutrophil function in an inflammatory milieu of rheumatoid arthritis. *J. Immunol. Res.* (2018). doi:10.1155/2018/8549329
496. Lauth, X. *et al.* M1 protein allows group a streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition. *J. Innate Immun.* (2009). doi:10.1159/000203645
497. Pennock, N. D. *et al.* T cell responses: naive to memory and everything in between. *AJP Adv. Physiol. Educ.* (2013). doi:10.1152/advan.00066.2013
498. Catron, D. M., Itano, A. A., Pape, K. A., Mueller, D. L. & Jenkins, M. K. Visualizing the first 50 hr of the primary immune response to a soluble antigen. *Immunity* (2004). doi:10.1016/j.immuni.2004.08.007
499. Miller, M. J., Safrina, O., Parker, I. & Cahalan, M. D. Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. *J. Exp. Med.* (2004). doi:10.1084/jem.20041236

500. van der Fits, L. *et al.* Imiquimod-Induced Psoriasis-Like Skin Inflammation in Mice Is Mediated via the IL-23/IL-17 Axis. *J. Immunol.* (2009). doi:10.4049/jimmunol.0802999
501. Hawkes, J. E., Gudjonsson, J. E. & Ward, N. L. The Snowballing Literature on Imiquimod-Induced Skin Inflammation in Mice: A Critical Appraisal. *Journal of Investigative Dermatology* (2017). doi:10.1016/j.jid.2016.10.024
502. Suzuki, H. *et al.* Imiquimod, a topical immune response modifier, induces migration of Langerhans cells. *J. Invest. Dermatol.* (2000). doi:10.1046/j.1523-1747.2000.00833.x
503. Hartwig, T. *et al.* Regulatory T Cells Restrain Pathogenic T Helper Cells during Skin Inflammation. *Cell Rep.* (2018). doi:10.1016/j.celrep.2018.12.012
504. Ueyama, A. *et al.* Mechanism of pathogenesis of imiquimod-induced skin inflammation in the mouse: A role for interferon-alpha in dendritic cell activation by imiquimod. *J. Dermatol.* (2014). doi:10.1111/1346-8138.12367
505. Horváth, S. *et al.* Methodological refinement of Aldara-induced psoriasiform dermatitis model in mice. *Sci. Rep.* (2019). doi:10.1038/s41598-019-39903-x
506. Haig, D. M., Hopkins, J. & Miller, H. R. P. Local immune responses in afferent and efferent lymph. *Immunology* (1999). doi:10.1046/j.1365-2567.1999.00681.x
507. Lee, P. Y., Wang, J.-X., Parisini, E., Dascher, C. C. & Nigrovic, P. A. Ly6 family proteins in neutrophil biology. *J. Leukoc. Biol.* (2013). doi:10.1189/jlb.0113014
508. Sato, T., Hongu, T., Sakamoto, M., Funakoshi, Y. & Kanaho, Y. Molecular Mechanisms of N-Formyl-Methionyl-Leucyl-Phenylalanine-Induced Superoxide Generation and Degranulation in Mouse Neutrophils: Phospholipase D Is Dispensable. *Mol. Cell. Biol.* (2013). doi:10.1128/mcb.00869-12
509. Bruhn, K. W., Dekitani, K., Nielsen, T. B., Pantapalangkoor, P. & Spellberg, B. Ly6G-mediated depletion of neutrophils is dependent on macrophages. *Results Immunol.* (2016). doi:10.1016/j.rinim.2015.12.001
510. Patel, D. F. *et al.* Neutrophils restrain allergic airway inflammation by limiting ILC2 function and monocyte-dendritic cell antigen presentation. *Sci. Immunol.* (2019). doi:10.1126/sciimmunol.aax7006
511. Azarov, I., Peskov, K., Helmlinger, G. & Kosinsky, Y. Role of T Cell-To-Dendritic cell chemoattraction in T Cell priming initiation in the lymph node: An agent-based modeling study. *Front. Immunol.* (2019). doi:10.3389/fimmu.2019.01289
512. Surcel, M. *et al.* Phenotypic changes of lymphocyte populations in psoriasiform dermatitis animal model. *Exp. Ther. Med.* (2018). doi:10.3892/etm.2018.6978
513. Tang, X., Basavarajappa, D., Haeggström, J. Z. & Wan, M. P2X 7 Receptor Regulates Internalization of Antimicrobial Peptide LL-37 by Human Macrophages That Promotes Intracellular Pathogen Clearance . *J. Immunol.* (2015). doi:10.4049/jimmunol.1402845
514. Hampton, H. R. & Chtanova, T. The lymph node neutrophil. *Semin. Immunol.* **28**, 129–136 (2016).
515. Gorlino, C. V. *et al.* Neutrophils Exhibit Differential Requirements for Homing Molecules in Their Lymphatic and Blood Trafficking into Draining Lymph Nodes. *J. Immunol.* **193**, 1966–1974 (2014).
516. Randolph, G. J., Angeli, V. & Swartz, M. A. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nature Reviews Immunology* (2005). doi:10.1038/nri1670
517. Rantakari, P. *et al.* The endothelial protein PLVAP in lymphatics controls the entry of lymphocytes and antigens into lymph nodes. *Nat. Immunol.* (2015).

doi:10.1038/ni.3101
518. Linderman, J. J. *et al.* Characterizing the Dynamics of CD4+ T Cell Priming within a Lymph Node. *J. Immunol.* (2010). doi:10.4049/jimmunol.0903117
519. Castell, S. D., Harman, M. F., Morón, G., Maletto, B. A. & Pistoresi-Palencia, M. C. Neutrophils which migrate to lymph nodes modulate CD4+ T cell response by a PD-L1 dependent mechanism. *Front. Immunol.* (2019). doi:10.3389/fimmu.2019.00105
520. Ahn, M. H. *et al.* Neutrophil extracellular traps may contribute to the pathogenesis in adult-onset still disease. *J. Rheumatol.* (2019). doi:10.3899/jrheum.181058
521. Tamassia, N. *et al.* Human neutrophils activated via TLR8 promote Th17 polarization through IL-23. *J. Leukoc. Biol.* (2019). doi:10.1002/JLB.MA0818-308R
522. Mitchell, T., Lo, A., Logan, M. R., Lacy, P. & Eitzen, G. Primary granule exocytosis in human neutrophils is regulated by Rac-dependent actin remodeling. *Am. J. Physiol. - Cell Physiol.* (2008). doi:10.1152/ajpcell.00239.2008
523. Hwang, S. A. *et al.* Lactoferrin enhanced efficacy of the BCG vaccine to generate host protective responses against challenge with virulent Mycobacterium tuberculosis. *Vaccine* (2007). doi:10.1016/j.vaccine.2007.07.005
524. Belkaid, Y. & Hand, T. W. Role of the microbiota in immunity and inflammation. *Cell* (2014). doi:10.1016/j.cell.2014.03.011
525. Faget, J. *et al.* Efficient and specific Ly6G+ cell depletion: A change in the current practices toward more relevant functional analyses of neutrophils. *bioRxiv* (2018). doi:10.1101/498881
526. Fanger, M. W., Shen, L., Graziano, R. F. & Guyre, P. M. Cytotoxicity mediated by human Fc receptors for IgG. *Immunol. Today* (1989). doi:10.1016/0167-5699(89)90234-X
527. Stark, M. A. *et al.* Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* (2005). doi:10.1016/j.immuni.2005.01.011
528. Mittrücker, H. W., Visekruna, A. & Huber, M. Heterogeneity in the Differentiation and Function of CD8+ T Cells. *Arch. Immunol. Ther. Exp. (Warsz).* (2014). doi:10.1007/s00005-014-0293-y
529. Pearce, E. L. *et al.* Control of Effector CD8+ T Cell Function by the Transcription Factor Eomesodermin. *Science (80-.).* (2003). doi:10.1126/science.1090148
530. Yen, H.-R. *et al.* Tc17 CD8 T Cells: Functional Plasticity and Subset Diversity. *J. Immunol.* (2009). doi:10.4049/jimmunol.0900368
531. Huber, M. *et al.* Th17-like developmental process leads to CD8+ Tc17 cells with reduced cytotoxic activity. *Eur. J. Immunol.* (2009). doi:10.1002/eji.200939412
532. Huber, M. *et al.* IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis. *J. Clin. Invest.* (2013). doi:10.1172/JCI63681
533. Srenathan, U., Steel, K. & Taams, L. S. IL-17+ CD8+ T cells: Differentiation, phenotype and role in inflammatory disease. *Immunol. Lett.* (2016). doi:10.1016/j.imlet.2016.05.001
534. Kondo, T., Takata, H., Matsuki, F. & Takiguchi, M. Cutting Edge: Phenotypic Characterization and Differentiation of Human CD8 + T Cells Producing IL-17 . *J. Immunol.* (2009). doi:10.4049/jimmunol.0801347
535. Kryczek, I. *et al.* Induction of IL-17 + T Cell Trafficking and Development by IFN-γ: Mechanism and Pathological Relevance in Psoriasis . *J. Immunol.* (2008). doi:10.4049/jimmunol.181.7.4733
536. Ono, S., Honda, T., Doi, H. & Kabashima, K. Concurrence of psoriasis vulgaris and atopic eczema in a single patient exhibiting different expression patterns of psoriatic

autoantigens in the lesional skin. *JAAD Case Reports* (2018). doi:10.1016/j.jdcr.2017.11.026

537. Bierkarre, H. *et al.* Differential expression of antimicrobial peptides in psoriasis and psoriatic arthritis as a novel contributory mechanism for skin and joint disease heterogeneity. *Scand. J. Rheumatol.* (2016). doi:10.3109/03009742.2015.1091497
538. Steel, K. J. A. *et al.* Polyfunctional, Proinflammatory, Tissue-Resident Memory Phenotype and Function of Synovial Interleukin-17A+CD8+ T Cells in Psoriatic Arthritis. *Arthritis Rheumatol.* (2019). doi:10.1002/art.41156
539. Bhat, P., Leggatt, G., Waterhouse, N. & Frazer, I. H. Interferon-γ derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. *Cell Death Dis.* (2017). doi:10.1038/cddis.2017.67
540. Chang, Y. *et al.* CD8 positive T cells express IL-17 in patients with chronic obstructive pulmonary disease. *Respir. Res.* (2011). doi:10.1186/1465-9921-12-43
541. Hayes, M. D., Ovcinnikovs, V., Smith, A. G., Kimber, I. & Dearman, R. J. The aryl hydrocarbon receptor: Differential contribution to T helper 17 and T cytotoxic 17 cell development. *PLoS One* **9**, (2014).
542. Tajima, M., Wakita, D., Satoh, T., Kitamura, H. & Nishimura, T. IL-17/IFN-γ double producing CD8 + T (Tc17/IFN-γ) cells: A novel cytotoxic T-cell subset converted from Tc17 cells by IL-12. *Int. Immunol.* (2011). doi:10.1093/intimm/dxr086
543. Satoh, T., Tajima, M., Wakita, D., Kitamura, H. & Nishimura, T. The development of IL-17/IFN-γ-double producing CTLs from Tc17 cells is driven by epigenetic suppression of Socs3 gene promoter. *Eur. J. Immunol.* (2012). doi:10.1002/eji.201142240
544. Chellappa, S. *et al.* CD8 + T Cells That Coexpress RORγt and T-bet Are Functionally Impaired and Expand in Patients with Distal Bile Duct Cancer . *J. Immunol.* (2017). doi:10.4049/jimmunol.1600061
545. Smyth, M. J. *et al.* IL-2 and IL-6 synergize to augment the pore-forming protein gene expression and cytotoxic potential of human peripheral blood T cells. *J. Immunol.* (1990).
546. Liu, S.-J. *et al.* Induction of a distinct CD8 Tnc17 subset by transforming growth factor-β and interleukin-6. *J. Leukoc. Biol.* (2007). doi:10.1189/jlb.0207111
547. Intlekofer, A. M. *et al.* Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat. Immunol.* (2005). doi:10.1038/ni1268
548. Curtis, M. M., Way, S. S. & Wilson, C. B. IL-23 Promotes the Production of IL-17 by Antigen-Specific CD8 T Cells in the Absence of IL-12 and Type-I Interferons. *J. Immunol.* (2009). doi:10.4049/jimmunol.0900939
549. Ciric, B., El-behi, M., Cabrera, R., Zhang, G.-X. & Rostami, A. IL-23 Drives Pathogenic IL-17-Producing CD8 + T Cells . *J. Immunol.* (2009). doi:10.4049/jimmunol.0900036
550. Sponaas, A. M. *et al.* PD1 is expressed on exhausted T cells as well as virus specific memory CD8+ T cells in the bone marrow of myeloma patients. *Oncotarget* (2018). doi:10.18632/oncotarget.25882
551. Ma, J. *et al.* PD1Hi CD8+ T cells correlate with exhausted signature and poor clinical outcome in hepatocellular carcinoma. *J. Immunother. Cancer* (2019). doi:10.1186/s40425-019-0814-7
552. Nakamoto, N. *et al.* Functional Restoration of HCV-Specific CD8 T Cells by PD-1 Blockade Is Defined by PD-1 Expression and Compartmentalization. *Gastroenterology* (2008). doi:10.1053/j.gastro.2008.02.033
553. Petrelli, A. *et al.* PD-1+CD8+ T cells are clonally expanding effectors in human

chronic inflammation. *J. Clin. Invest.* (2018). doi:10.1172/JCI96107
554. Blackburn, S. D. *et al.* Tissue-Specific Differences in PD-1 and PD-L1 Expression during Chronic Viral Infection: Implications for CD8 T-Cell Exhaustion. *J. Virol.* (2010). doi:10.1128/jvi.01579-09
555. Zhang, X. & Xu, W. Neutrophils diminish T-cell immunity to foster gastric cancer progression: The role of GM-CSF/PD-L1/PD-1 signalling pathway. *Gut* (2017). doi:10.1136/gutjnl-2017-313923
556. Ortega, C. *et al.* IL-17-producing CD8 + T lymphocytes from psoriasis skin plaques are cytotoxic effector cells that secrete Th17-related cytokines . *J. Leukoc. Biol.* (2009). doi:10.1189/jlb.0109046
557. Liang, Y., Pan, H. F. & Ye, D. Q. Tc17 cells in immunity and systemic autoimmunity. *Int. Rev. Immunol.* **34**, 318–331 (2015).
558. Hayes, M. D., Ovcinnikovs, V., Smith, A. G., Kimber, I. & Dearman, R. J. The aryl hydrocarbon receptor: Differential contribution to T helper 17 and T cytotoxic 17 cell development. *PLoS One* (2014). doi:10.1371/journal.pone.0106955
559. Pick, J. *et al.* CTLA-4 (CD152) enhances the Tc17 differentiation program. *Eur. J. Immunol.* (2014). doi:10.1002/eji.201343497
560. Lehmann, J. S., Zhao, A., Sun, B., Jiang, W. & Ji, S. Multiplex cytokine profiling of stimulated mouse splenocytes using a cytometric bead-based immunoassay platform. *J. Vis. Exp.* (2017). doi:10.3791/56440
561. Zhu, Y. *et al.* T-bet and Eomesodermin Are Required for T Cell-Mediated Antitumor Immune Responses. *J. Immunol.* (2010). doi:10.4049/jimmunol.1000749
562. Yang, R. *et al.* IL-6 IL-6 promotes the differentiation of a subset of naive CD8+ T cells into IL-21-producing B helper CD8+ T cells. *J. Exp. Med.* (2016). doi:10.1084/JEM.20160417
563. Mihailovic, P. M. *et al.* The cathelicidin protein CRAMP is a potential atherosclerosis self-antigen in ApoE(-/-) mice. *PLoS One* (2017). doi:10.1371/journal.pone.0187432
564. Liu, X. *et al.* RORG agonists enhance the sustained antitumor activity through intrinsic TC17 cytotoxicity and TC1 recruitment. *Cancer Immunol. Res.* (2019). doi:10.1158/2326-6066.CIR-18-0714
565. Flores-Santibáñez, F. *et al.* CD73-mediated adenosine production promotes stem cell-like properties in mouse Tc17 cells. *Immunology* (2015). doi:10.1111/imm.12529
566. Flores-Santibáñez, F. *et al.* In vitro-generated Tc17 cells present a memory phenotype and serve as a reservoir of Tc1 cells in vivo. *Front. Immunol.* (2018). doi:10.3389/fimmu.2018.00209
567. Nanjappa, S. G. *et al.* Antifungal Tc17 cells are durable and stable, persisting as long-lasting vaccine memory without plasticity towards IFNγ cells. *PLoS Pathog.* (2017). doi:10.1371/journal.ppat.1006356
568. Filippi, C. M. *et al.* Transforming growth factor-β suppresses the activation of cd8 + t-cells when naive but promotes their survival and function once antigen experienced: a two-faced impact on autoimmunity. *Diabetes* (2008). doi:10.2337/db08-0609
569. Gartlan, K. H. *et al.* Tc17 cells are a proinflammatory, plastic lineage of pathogenic CD8+ T cells that induce GVHD without antileukemic effects. *Blood* (2015). doi:10.1182/blood-2015-01-622662
570. Dwivedi, V. P. *et al.* Transforming growth factor-β protein inversely regulates in vivo differentiation of interleukin-17 (IL-17)-producing CD4 + and CD8 + T cells. *J. Biol. Chem.* (2012). doi:10.1074/jbc.C111.327627

571. Cheuk, S. *et al.* Epidermal Th22 and Tc17 Cells Form a Localized Disease Memory in Clinically Healed Psoriasis. *J. Immunol.* (2014). doi:10.4049/jimmunol.1302313
572. Di Meglio, P. *et al.* Targeting CD8+ T cells prevents psoriasis development. *J. Allergy Clin. Immunol.* (2016). doi:10.1016/j.jaci.2015.10.046
573. Renavikar, P. S., Crawford, M. P., Sinha, S. & Karandikar, N. J. Human Tc17 cells harbor potent immune suppressive potential, whereas Tc1 cells lack suppressive ability. *J. Immunol.* **202**, (2019).
574. Sinha, S., Itani, F. R. & Karandikar, N. J. Immune regulation of multiple sclerosis by CD8+ T cells. *Immunologic Research* (2014). doi:10.1007/s12026-014-8529-9
575. Song, M. H., Nair, V. S. & Oh, K. I. Vitamin C enhances the expression of IL17 in a Jmjd2-dependent manner. *BMB Rep.* (2017). doi:10.5483/BMBRep.2017.50.1.193
576. LL-37 suppresses apoptosis of dermal fibroblasts in patients with systemic sclerosis. *J. Am. Acad. Dermatol.* (2012). doi:10.1016/j.jaad.2011.11.282
577. Motran, C. C., Molinder, K. M., Liu, S. D., Poirier, F. & Miceli, M. C. Galectin-1 functions as a Th2 cytokine that selectively induces Th1 apoptosis and promotes Th2 function. *Eur. J. Immunol.* (2008). doi:10.1002/eji.200838295
578. Coomes, S. M. *et al.* CD4 + Th2 cells are directly regulated by IL-10 during allergic airway inflammation. *Mucosal Immunol.* (2017). doi:10.1038/mi.2016.47
579. Elyaman, W. *et al.* IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc. Natl. Acad. Sci. U. S. A.* (2009). doi:10.1073/pnas.0812530106
580. Coccia, M. *et al.* IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4 + Th17 cells. *J. Exp. Med.* (2012). doi:10.1084/jem.20111453
581. Smith, K. A. Interleukin-2: Inception, impact, and implications. *Science (80-.).* (1988). doi:10.1126/science.3131876
582. Aarts, C. E. M. *et al.* Neutrophils as Suppressors of T Cell Proliferation: Does Age Matter? *Front. Immunol.* (2019). doi:10.3389/fimmu.2019.02144
583. Kelly, E., Won, A., Refaeli, Y. & Van Parijs, L. IL-2 and Related Cytokines Can Promote T Cell Survival by Activating AKT. *J. Immunol.* (2002). doi:10.4049/jimmunol.168.2.597
584. Khattar, M. *et al.* Interleukin-21 is a critical regulator of CD4 and CD8 T cell survival during priming under interleukin-2 deprivation conditions. *PLoS One* (2014). doi:10.1371/journal.pone.0085882
585. Scapini, P. *et al.* G-CSF-stimulated neutrophils are a prominent source of functional BLyS. *J. Exp. Med.* (2003). doi:10.1084/jem.20021343
586. Ueda, R. *et al.* Interaction of natural killer cells with neutrophils exerts a significant antitumor immunity in hematopoietic stem cell transplantation recipients. *Cancer Med.* (2016). doi:10.1002/cam4.550
587. Huard, B. *et al.* APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa. *J. Clin. Invest.* (2008). doi:10.1172/JCI33760
588. Chiewchengchol, D. *et al.* The protective effect of GM-CSF on serum-induced neutrophil apoptosis in juvenile systemic lupus erythematosus patients. *Clin. Rheumatol.* (2015). doi:10.1007/s10067-014-2800-2
589. Tabarkiewicz, J., Pogoda, K., Karczmarczyk, A., Pozarowski, P. & Giannopoulos, K. The Role of IL-17 and Th17 Lymphocytes in Autoimmune Diseases. *Archivum Immunologiae et Therapiae Experimentalis* (2015). doi:10.1007/s00005-015-0344-z

590. Raza, K. *et al.* Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. *Arthritis Res. Ther.* (2005). doi:10.1186/ar1733
591. Chiang, C. C., Cheng, W. J., Korinek, M., Lin, C. Y. & Hwang, T. L. Neutrophils in Psoriasis. *Frontiers in Immunology* (2019). doi:10.3389/fimmu.2019.02376
592. Bals, R., Weiner, D. J. & Wilson, J. M. The innate immune system in cystic fibrosis lung disease. *Journal of Clinical Investigation* (1999). doi:10.1172/JCI6277
593. Nijnik, A. & Hancock, R. Host defence peptides: antimicrobial and immunomodulatory activity and potential applications for tackling antibiotic-resistant infections. *Emerg. Health Threats J.* (2009). doi:10.3134/ehtj.09.001

Appendix

Gene	Log2 FC	P-value
Abcf1-mRNA	0.0665	0.610
Abl1-mRNA	-0.353	0.212
Adal-mRNA	0.0626	0.793
Ahr-mRNA	1.72	0.016
Arhgdib-mRNA	-0.0253	0.863
Atg16l1-mRNA	0.13	0.509
Atm-mRNA	0.199	0.981
B2m-mRNA	-0.725	0.000
Batf-mRNA	0.61	0.017
Bax-mRNA	-0.0898	0.620
Bcap31-mRNA	-0.314	0.100
Bcl2-mRNA	-0.631	0.037
Bcl3-mRNA	-1.46	0.014
Bcl6-mRNA	0.132	0.972
Bid-mRNA	0.0166	0.998
Bst2-mRNA	-0.345	0.345
Btla-mRNA	0.394	0.109
C1qbp-mRNA	0.0361	0.825
Casp2-mRNA	0.21	0.159
Casp3-mRNA	-0.163	0.163
Casp8-mRNA	-0.0128	0.944
Ccl25-mRNA	-0.139	0.692
Ccl3-mRNA	-0.891	0.683
Ccl4-mRNA	-1.86	0.399
Ccr4-mRNA	-0.147	0.790
Ccr6-mRNA	0.411	0.857
Ccr7-mRNA	-0.91	0.010
Ccr8-mRNA	-0.235	0.066
Cd164-mRNA	-0.0409	0.696
Cd19-mRNA	0.366	0.914
Cd2-mRNA	-0.519	0.017

Gene	Log2 FC	P-value
Cd226-mRNA	-0.377	0.199
Cd247-mRNA	-0.357	0.060
Cd27-mRNA	-0.511	0.025
Cd274-mRNA	-0.919	0.020
Cd28-mRNA	0.492	0.054
Cd3d-mRNA	-0.24	0.193
Cd3e-mRNA	0.105	0.538
Cd3eap-mRNA	-0.157	0.979
Cd4-mRNA	-0.354	0.042
Cd40lg-mRNA	-1.21	0.029
Cd44-mRNA	-0.358	0.293
Cd48-mRNA	0.0589	0.604
Cd5-mRNA	0.198	0.183
Cd53-mRNA	-0.0504	0.678
Cd55-mRNA	-0.652	0.894
Cd6-mRNA	0.339	0.019
Cd69-mRNA	-0.401	0.092
Cd74-mRNA	-0.553	0.025
Cd79b-mRNA	-0.296	0.091
Cd81-mRNA	0.175	0.297
Cd82-mRNA	-0.36	0.100
Cd83-mRNA	-0.741	0.031
Cd9-mRNA	-1.13	0.015
Cd96-mRNA	-0.704	0.934
Cd97-mRNA	-1.83	0.703
Cd99-mRNA	0.000853	0.997
Cdkn1a-mRNA	0.677	0.922
Cebpb-mRNA	0.00506	0.998
Chuk-mRNA	0.0575	0.722
Ciita-mRNA	0.00165	1.000
Cish-mRNA	-1.3	0.656

Table A1: List of differentially expressed genes in response to mCRAMP (*Abcf1* - *Cish*)

Gene	Log2 FC	P-value
Cradd-mRNA	0.12	0.988
Crlf2-mRNA	-0.406	0.047
Ctla4-mRNA	0.712	0.003
Ctnnb1-mRNA	-0.172	0.029
Ctsc-mRNA	0.385	0.018
Ctss-mRNA	-1.08	0.005
Cxcr3-mRNA	-0.672	0.032
Cxcr4-mRNA	-0.0969	0.658
Cxcr5-mRNA	1.14	0.850
Cxcr6-mRNA	-1.09	0.896
Dpp4-mRNA	-0.443	0.953
Ets1-mRNA	-0.29	0.109
Fadd-mRNA	1.01	0.047
Fcgr2b-mRNA	-0.0955	0.982
Fkbp5-mRNA	-0.073	0.994
Folr4-mRNA	-0.614	0.015
Foxp3-mRNA	-0.564	0.082
Fyn-mRNA	-0.143	0.386
Gata3-mRNA	-1.29	0.616
Gfi1-mRNA	-0.581	0.148
Gm10499-mRNA	-0.199	0.484
Gpr183-mRNA	-0.198	0.612
H2-Aa-mRNA	-0.46	0.030
H2-Ab1-mRNA	-0.149	0.419
H2-DMa-mRNA	-0.616	0.000
H2-DMb2-mRNA	-0.88	0.003
H2-Eb1-mRNA	-0.176	0.458
H2-K1-mRNA	-0.875	0.005
H2-Ob-mRNA	-0.0911	0.779
Hcst-mRNA	-0.561	0.902
Hif1a-mRNA	0.694	0.006

Gene	Log2 FC	P-value
Icam1-mRNA	-0.367	0.908
Icam2-mRNA	-0.188	0.124
Icos-mRNA	-0.28	0.031
Ifi35-mRNA	-0.545	0.024
Ifih1-mRNA	-0.813	0.751
Ifit2-mRNA	0.00401	0.999
Ifnar1-mRNA	-0.271	0.166
Ifnar2-mRNA	0.0116	0.956
Ifngr1-mRNA	-0.456	0.092
Ifngr2-mRNA	-1.01	0.008
Igf2r-mRNA	0.338	0.306
Ikbkap-mRNA	0.382	0.187
Ikbkb-mRNA	0.35	0.967
Ikbke-mRNA	-1.02	0.009
Ikbkg-mRNA	0.0683	0.713
Ikzf1-mRNA	-0.03	0.844
Ikzf2-mRNA	0.111	0.850
Ikzf3-mRNA	-0.117	0.470
Ikzf4-mRNA	-1.49	0.002
Il10ra-mRNA	-0.0575	0.987
Il10rb-mRNA	-0.266	0.146
Il12rb1-mRNA	0.129	0.970
Il16-mRNA	-0.0678	0.711
Il17f-mRNA	0.522	0.850
Il17ra-mRNA	-0.574	0.030
Il18r1-mRNA	-0.348	0.959
Il1rap-mRNA	-0.159	0.527
Il2-mRNA	-1.7	0.066
Il21-mRNA	0.789	0.737
Il21r-mRNA	-0.518	0.824
Il27ra-mRNA	0.164	0.215

Table A1: List of differentially expressed genes in response to mCRAMP (*Cradd* – *Il27ra*)

Gene	Log2 FC	P-value
Il2ra-mRNA	0.449	0.080
Il2rb-mRNA	-0.122	0.418
Il2rg-mRNA	-0.249	0.033
Il4ra-mRNA	0.0247	0.886
Il6ra-mRNA	-0.0916	0.575
Il6st-mRNA	0.442	0.069
Il7r-mRNA	-0.619	0.116
Ilf3-mRNA	0.126	0.541
Irak1-mRNA	-0.0857	0.570
Irak2-mRNA	-0.378	0.915
Irak4-mRNA	-0.237	0.021
Irf1-mRNA	-0.791	0.024
Irf3-mRNA	0.17	0.572
Irf4-mRNA	0.485	0.049
Irf8-mRNA	-1.44	0.033
Irgm1-mRNA	-0.842	0.117
Itga4-mRNA	-0.579	0.909
Itga6-mRNA	-0.531	0.031
Itgal-mRNA	0.402	0.043
Itgb1-mRNA	0.314	0.145
Itgb2-mRNA	-0.13	0.404
Jak1-mRNA	0.0474	0.714
Jak2-mRNA	-0.724	0.007
Jak3-mRNA	0.303	0.170
Klrd1-mRNA	0.312	0.962
Lck-mRNA	0.0312	0.842
Lcp2-mRNA	-0.492	0.351
Lef1-mRNA	-0.256	0.047
Litaf-mRNA	-0.643	0.932
Lta-mRNA	-0.895	0.144
Ltb-mRNA	-1.71	0.000

Gene	Log2 FC	P-value
Maf-mRNA	0.847	0.005
Map4k1-mRNA	0.182	0.516
Map4k2-mRNA	-0.644	0.015
Map4k4-mRNA	0.351	0.219
Mapk1-mRNA	0.073	0.601
Mapk14-mRNA	0.534	0.003
Mapkapk2-mRNA	-0.159	0.369
Mbp-mRNA	-0.953	0.026
Mif-mRNA	0.0872	0.566
Ms4a1-mRNA	-0.165	0.429
Mx1-mRNA	0.471	0.830
Myd88-mRNA	0.0116	0.870
Ncf4-mRNA	0.303	0.242
Nfatc1-mRNA	0.144	0.266
Nfatc2-mRNA	0.264	0.198
Nfatc3-mRNA	0.143	0.435
Nfil3-mRNA	0.293	0.973
Nfkb1-mRNA	-0.336	0.029
Nfkb2-mRNA	-0.0457	0.758
Nfkbia-mRNA	-0.0753	0.680
Nfkbiz-mRNA	-0.0385	0.885
Notch1-mRNA	0.182	0.354
Notch2-mRNA	-0.228	0.380
Npc1-mRNA	-0.0913	0.669
Nt5e-mRNA	0.538	0.049
Pdcd1-mRNA	0.186	0.262
Pdcd1lg2-mRNA	0.688	0.011
Pdcd2-mRNA	-0.237	0.369
Pdgfb-mRNA	-0.0581	0.556
Pecam1-mRNA	-1.07	0.916
Phlpp1-mRNA	0.325	0.962

Table A1: List of differentially expressed genes in response to mCRAMP (*Il2ra* – *Phlpp1*)

Gene	Log2 FC	P-value
Phlpp2-mRNA	0.754	0.933
Pml-mRNA	-0.172	0.548
Pou2f2-mRNA	-0.75	0.039
Prim1-mRNA	0.146	0.496
Prkcd-mRNA	-0.204	0.310
Psmb10-mRNA	-0.654	0.831
Psmb5-mRNA	0.0602	0.502
Psmb7-mRNA	-0.0983	0.108
Psmb9-mRNA	-0.777	0.021
Psmc2-mRNA	-0.006	0.882
Psmd7-mRNA	0.132	0.362
Ptger4-mRNA	0.155	0.526
Ptpn2-mRNA	-0.0134	0.911
Ptpn22-mRNA	-0.286	0.054
Ptpn6-mRNA	-0.49	0.019
Ptprc-mRNA	-0.052	0.713
Rae1-mRNA	-0.0563	0.741
Rela-mRNA	0.132	0.168
Relb-mRNA	-0.093	0.395
Rorc-mRNA	0.621	0.077
Runx1-mRNA	-0.0958	0.579
S100a8-mRNA	-0.132	0.977
S100a9-mRNA	0.041	0.991
Sell-mRNA	-1.76	0.000
Sh2d1a-mRNA	-0.211	0.516
Sigirr-mRNA	-0.109	0.484
Ski-mRNA	-0.7	0.016
Slamf1-mRNA	-0.493	0.008
Slamf7-mRNA	-0.152	0.546
Smad3-mRNA	0.503	0.148
Smad5-mRNA	0.198	0.978

Gene	Log2 FC	P-value
Socs1-mRNA	-0.801	0.038
Socs3-mRNA	-1.04	0.026
Spn-mRNA	-0.108	0.659
Stat1-mRNA	-0.686	0.005
Stat3-mRNA	0.131	0.330
Stat4-mRNA	-0.134	0.668
Stat5a-mRNA	-0.0707	0.741
Stat5b-mRNA	-0.0522	0.568
Stat6-mRNA	-0.0805	0.218
Tagap-mRNA	-0.504	0.068
Tap1-mRNA	-0.377	0.189
Tapbp-mRNA	-1.03	0.003
Tbk1-mRNA	0.148	0.329
Tcf4-mRNA	0.272	0.392
Tcf7-mRNA	-1.12	0.007
Tfrc-mRNA	0.737	0.018
Tgfb1-mRNA	-0.0688	0.617
Tgfbr1-mRNA	0.135	0.117
Tgfbr2-mRNA	-0.209	0.219
Thy1-mRNA	-0.954	0.002
Tigit-mRNA	0.29	0.956
Tlr1-mRNA	-0.422	0.962
Tmem173-mRNA	-0.0108	0.941
Tnf-mRNA	-0.592	0.059
Tnfaip3-mRNA	-0.458	0.102
Tnfrsf13b-mRNA	0.109	0.652
Tnfrsf14-mRNA	-0.024	0.878
Tnfrsf1b-mRNA	-0.759	0.005
Tnfrsf4-mRNA	-0.421	0.083
Tnfrsf9-mRNA	-0.922	0.039
Tnfsf10-mRNA	-0.784	0.879

Table A1: List of differentially expressed genes in response to mCRAMP (*Phlpp2* – *Tnfsf10*)

Gene	Log2 FC	P-value
Tnfsf11-mRNA	-0.503	0.929
Tnfsf14-mRNA	-2.05	0.680
Tnfsf8-mRNA	-0.168	0.966
Tollip-mRNA	0.0267	0.844
Traf1-mRNA	-0.715	0.029
Traf2-mRNA	0.163	0.980
Traf3-mRNA	0.289	0.174
Traf4-mRNA	-0.477	0.968
Traf5-mRNA	-0.367	0.917
Traf6-mRNA	0.0838	0.642
Trp53-mRNA	0.0241	0.860
Tyk2-mRNA	0.264	0.977
Ube2l3-mRNA	-0.0228	0.667
Xbp1-mRNA	-0.395	0.003
Xcl1-mRNA	-1.95	0.000
Zap70-mRNA	0.167	0.279
Zbtb7b-mRNA	-0.254	0.127
Zeb1-mRNA	0.143	0.221

Table A1: List of differentially expressed genes in response to mCRAMP (*Tnfsf11* – *Zeb1*)